MECHANICAL, OPTICAL, AND WATER VAPOR BARRIER PROPERTIES OF CANOLA PROTEIN ISOLATE-BASED EDIBLE FILMS

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By

Chang Chang

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ABSTRACT

Biodegradable edible films are both economically and environmentally important to the food industry as packaging and coating materials, as the industry seeks to find a replacement to traditional petroleum-derived synthetic polymers. The overall goal of this thesis was to design a canola protein isolate (CPI)-based biodegradable and edible film that provides excellent mechanical, optical and water vapor barrier properties. A better understanding of the potential of CPI for use as a film-forming ingredient could lead to enhanced utilization and value of the protein for food and non-food applications.

In study one, the mechanical, optical and water vapor barrier properties of CPI-based films were investigated as a function of protein (5.0% and 7.5% w/w) and glycerol (30%, 35%, 40%, 45%, and 50% w/w of CPI) concentrations. Overall, as the glycerol concentration increased for the 5.0% and 7.5% CPI-based films, mechanical strength and flexibility decreased and increased, respectively. Film strength was also found to increase at the higher protein concentration; however corresponding changes to film flexibility differed depending on the testing method used. For instance, puncture deformation testing indicated that film flexibility was reduced as the CPI concentration was raised, whereas tensile elongation testing indicated no change in extensibility between the two CPI concentrations. Film transparency was found to increase with increasing levels of glycerol and decreasing levels of CPI, whereas water vapor permeability was found to increase with increasing levels of both glycerol and protein.

In study two, mechanical, optical and vapor barrier properties of CPI-based films were evaluated as a function of plasticizer-type (50% (w/w of CPI), glycerol, sorbitol, polyethylene glycol 400 (PEG-400)) and fixative condition (0% and 1% (w/w of CPI), genipin). CPI films prepared with sorbitol were significantly stronger than films with PEG-400, followed by films with glycerol, whereas the flexibility of CPI-based films with glycerol was higher than films with PEG-400, followed by films with sorbitol. In all cases, films prepared with genipin were stronger and less malleable than un-cross linked films. CPI films with glycerol were more transparent than films with sorbitol, followed by films with PEG-400, and the addition of genipin significantly increased the opacity of CPI films. CPI films prepared with glycerol also showed poorer water vapor barrier property than films with PEG-400, followed by films with sorbitol, however, no differences were observed in the presence and absence of genipin.

In summary, as the plasticizer concentration increased or protein concentration decreased, CPI films became weaker, more flexible and clearer; however their water vapor barrier properties became poorer as both plasticizer and protein concentration increased. Moreover, CPI films with sorbitol and genipin were found to be stronger, less malleable and permeable to moisture than CPI films with or without genipin, and in the presence of glycerol or PEG-400. Overall, CPI could be considered as a potential material for the development of biodegradable edible packaging in the future.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

CPI	Canola protein isolate
Gly	Glycerol
GP	Genipin
Sor	Sorbitol
PEG-400	Polyethylene glycol 400
TS	Tensile strength
TE	Tensile elongation
E	Elastic modulus
PS	Puncture strength
PD	Puncture deformation
SEM	Scanning electron microscopy
WVP	Water vapor permeability
RH	Relative humidity
MTGase	Transglutaminase
PVC	Polyvinyl chloride
PTFE	Polytetrafluoroethylene
$WVTR_m$	Water vapor transmission rate
L	Thickness of the film (mm)
P_{w1}	Water vapor partial pressure at the film inner surface
P_{w2}	Water vapor partial pressure at the film outer surface
P_T	Total atmospheric pressure
P_{w0}	Partial pressure of water vapor at the air of the surface of the $Mg(NO_3)_2$ solution
N_w	The measured value of $WVTR_m$
c	Total molar concentration of the air and water vapor
D	Diffusivity of water vapor through the air at 25 ºC
UV	Ultraviolet
SPI	Soy protein isolate
WG	Wheat gluten
LPC	Lentil protein concentrate

FPI	Faba bean protein isolate
RP	Rapeseed protein
PPI	Pea protein isolate
ISFP	Sunflower protein isolate
pI	Isoelectric point
TSM	Total soluble matter
DSC	Differential scanning calorimetry
ANOVA	Analysis of variance
Tg	Glass transition temperature
db	Dry basis
A.nm	Absorbance nanometer
M	Molar
U	Unit
w	Weight
g	Gram
nm	Nanometer
mm	Millimeter
cm	Centimeter
m	Meter
s	Second
min	Minute
h	Hour
d	Day
S	Svedberg unit
rpm	Rotations per minute
N	Newton
MPa	Megapascal
kPa	Kilopascal
Pa	Pascal
kDa	Kilodalton

1. INTRODUCTION

1.1 Overview

Over the past decade, there has been an increased interest surrounding the use of biodegradable edible films by the food packaging industry as a way to reduce their environmental footprint (Vargas et al., 2008; Gomez-Estaca et al., 2009; Janjarasskul & Krochta, 2010). As such, researchers have been investigating the use of natural biopolymer-based materials (e.g., protein-, polysaccharide- and lipid-based) as an alternative to synthetic petroleum-based polymers. These biopolymer-based packages are considered to be both economical and bio-friendly. Furthermore, depending on the composition, films may display excellent barrier properties to moisture, gases and aromas; have the ability to carry and deliver various additives (e.g., antimicrobial agents and antioxidants) for extended product shelf-life or improved quality; or help improve a product's structural integrity and handling characteristics (Psomiadou et al., 1996; Krochta & De Mulder-Johnston, 1997; Han & Gennadios, 2005). Protein- and polysaccharide-based materials tend to form films with excellent mechanical properties and gas barrier properties, but offer poor moisture control (Kester & Fennema, 1986; Baldwin et al., 1995; Vargas et al., 2008; Janjarasskul & Krochta, 2010). In contrast, lipid-based films tend to have excellent moisture barrier property, but have poor mechanical and gas barrier properties (Greener & Fennema, 1989; Janjarasskul & Krochta, 2010). The formation of edible films using proteins from plant sources has been limited, but may be advantageous to those from animal sources, because of their low cost, and perceived safety concerns (e.g., prions) by consumers or dietary restrictions over consuming animal-derived products (Uppstrom, 1995; Gennadios, 2002). Films have been prepared previously using proteins from plant sources, such as soy (Cho & Rhee, 2004), sunflower (Orliac et al., 2002), lentil (Bamdad et al., 2006), faba bean (Saremnezhad et al., 2011), pea (Kowalczyk & Baraniak, 2011), and rapeseed (Jang et al., 2011).

In an effort to tailor the mechanical and barrier properties of protein-based films, various factors have been previously explored including protein concentration (Jang et al., 2011),

plasticizer concentration/type (Gennadios et al., 1996; Cao et al., 2009; Mikkonen, et al., 2009), film forming conditions (e.g., pH, temperature, and the presence of salts) (Kowalczyk & Baraniak, 2011; Saremnezhad et al., 2011), and the addition of cross linking agents (Tang et al., 2005; Tang & Jiang, 2007; Gonzalez et al., 2011). To improve the flexibility and to overcome brittleness of films, plasticizers (e.g., glycerol, sorbitol, and polyethylene glycol 400 (PEG-400)) are typically added to soften the structure (Gennadios et al., 1996; Cao et al., 2009; Mikkonen et al., 2009). The effectiveness is dependent on the composition, size, and shape of plasticizer used (Sothornvit & Krochta, 2001).

Moreover, the formation of cross links by the addition of enzymatic or chemical fixatives has also been shown to influence film properties. For instance, genipin (GP), a natural chemical cross linking agent extracted from *Gardenia Jasminoides Ellis* fruit has showed some promise, as it can result in cross links of similar strengths as glutaraldehyde but is 10,000 times less cytotoxic (Song & Zhang, 2009). GP reacts with the primary amines (mainly lysine) within the protein to form both inter- and intramolecular cross links. Once reacted, a dark blue pigment develops (Touyama et al., 1994). Recently, genipin cross linking was used to fix films derived from chitosan (Jin et al., 2004), silk fibroin and sericin (Motta et al., 2011), and soy protein (Gonzalez et al., 2011). Transglutaminase, a natural enzymatic cross linking agent, has also been widely used to improve the properties of edible films, such as from soy (Tang et al., 2005; Tang & Jiang, 2007) and wheat gluten (Tang & Jiang, 2007).

Canola proteins have received some interests over the past few decades in terms of their functional attributes (Aluko & McIntosh, 2001; Yoshie-Stark et al., 2008), however despite this, protein products have not gained any traction as a new food ingredient until recently. A few companies (e.g., BioExx Specialty Proteins (Toronto, ON, Canada) and Burcon NutraSciences (Vancouver, BC, Canada)) are looking to start moving canola protein ingredients into the marketplace. Canola (*Brassicaceae spp.*) is primarily grown for its oil content to be used for cooking and biodiesel purposes (Wu & Muir, 2008). Once the oil is pressed, the remaining meal (high in protein and fiber) is typically used in feed applications (Uruakpa & Arntfield, 2005). In order to improve the viability of the canola industry, proteins are now being extracted from the meal as a value-added by-product for both food and non-food applications. Canola proteins are dominated by a salt-soluble globulin protein (cruciferin, 11S, molecular weight of 300 kDa) and a water-soluble albumin protein (napin, 2S, molecular weight of 12.5-15 kDa) (Wanasundara,

2011).

The overall goal of this thesis was to design a canola protein isolate-based film that offers excellent mechanical, optical and moisture barrier properties. Specifically, protein concentration, plasticizer-type and concentration, and the presence of genipin were tested for their effects on film properties. Enhanced utilization of canola proteins may increase their integration into the vegetable protein ingredient market.

1.2 Objectives

The overarching goal of this research project was to a create canola protein isolate (CPI)-based film that provides excellent water vapor barrier, optical and mechanical properties. The specific objectives of this research were: a) to examine the mechanical, optical, and water vapor barrier properties of CPI-based films as a function of both protein and glycerol concentrations; and b) to evaluate the effects of plasticizer-type and genipin on the mechanical, optical and water vapor barrier properties of films. Information from the two studies will help better our understanding of how changes to film forming solution formulations can help tailor film properties.

1.3 Hypotheses

The following hypotheses were tested as part of this research: a) film prepared at higher CPI concentration and lower glycerol level will form stronger, but less flexible films, with higher opaqueness and greater water vapor permeability; and b) films prepared with plasticizers of higher molecular mass will be weaker, but more flexible, with higher opacity and lower water vapor permeability; and c) films prepared in the presence of genipin will be stronger, less flexible, with higher opacity and lower water vapor permeability than films without a fixative.

2. LITERATURE REVIEW

2.1 Biodegradable edible films

Biodegradable edible films are both economically and environmentally important to the food industry in terms of packaging and coating materials. Traditional petroleum-derived synthetic materials used in consumer packaging create tremendous demands in landfills, the environment and consumer health. As such, research activities surrounding biodegradable edible packaging have been increased substantially over the past decade as the food industry attempts to find an alternative to synthetic petroleum-based polymers using bio-based materials, such as proteins, polysaccharides, and lipids (Vargas et al., 2008; Gomez-Estaca et al., 2009; Janjarasskul & Krochta, 2010). In addition to the alleviated environmental impacts, depending on the materials selected, films may have the added advantages of being edible and/or being used as a controlled delivery system for bioactive (e.g., sodium alginate-gellan gum coating containing N-acetylcysteine and glutathione (Rojas-Grau et al., 2007)) or antimicrobial (e.g., hydroxyl propyl methyl cellulose-based film containing nisin (Sebti & Coma, 2002)) compounds to maintain product quality and extend shelf-life (Ou et al., 2004; Han & Gennadios, 2005). Typically, biodegradable edible films tend to be self-supporting and <250 microns thick, used to encase a product or to separate heterogeneous prepared food products to keep ingredients separate (e.g., to inhibit or control moisture transfer) (Krochta & De Mulder-Johnston, 1997; Janjarasskul & Krochta, 2010). Edible materials within films are classified more as additives than ingredients, as they have no significant nutritional value (Debeaufort et al., 1998). The films are required to be relatively tasteless to help prevent consumer detection (Contreras-Medellin & Labuza, 1981). Implementation of biodegradable edible packaging by the food industry will help offset demands on our landfills and the environment, and enhance consumers' health and wellness (e.g., due to reduced levels of potential chemicals that could leach into our foods), and improve product quality. Other advantages for using the biopolymer-based films may include: transparency, mechanical strength, barrier properties (moisture and gases) and their use in controlled delivery applications (Debeaufort et al., 1998; Janjarasskul & Krochta, 2010; Falguera

et al., 2011). These characteristics can be tailored through material selection, biopolymer characteristics (e.g., concentration), solvent (e.g., pH and the presence of salts), the environment (e.g., relative humidity and temperature) and processing techniques. Film performance is typically assessed based on its mechanical properties, gas permeability, water vapor permeability, opacity, and moisture sorption property, based on common testing methods (Table 2.1).

2.1.1 Film materials

Biodegradable edible films are generally classified as being comprised of either lipids (e.g., solid fats, waxes, or resins) or biopolymers (e.g., proteins or polysaccharides); each has its own advantages and disadvantages (Table 2.2). Protein-based (e.g., gelatin, whey, soy and corn zein) and polysaccharide-based (e.g., alginate, carrageenan, chitosan and pectin) materials tend to form films with excellent mechanical properties and gas barrier properties, but have issues relating to moisture control due to the hydrophilic nature of the materials (Baldwin et al., 1995; Vargas et al., 2008; Janjarasskul & Krochta, 2010). In contrast, lipid-based (e.g., beeswax) films tend to display poor mechanical integrity and gas barrier properties, but provide excellent moisture control (Greener & Fennema, 1989; Janjarasskul & Krochta, 2010). In order to overcome deficiencies associated with lipid-based or biopolymer-based films, research is now primarily focused on composite films involving both. Optimization of film formulation is essential in order to balance the positive and negative attributes of each material.

Protein-based edible films developed from wheat gluten, casein, whey protein, and gelatin can be expensive, and as such, other plant protein materials have been explored for their potentialities to develop biodegradable edible films (Table 2.3). Of particular interest, is protein-rich meals left over from oil seed pressing (e.g., from soybean and canola) which tend to be low cost, abundant and have a high nutritional value.

2.1.2 Film preparation

Biopolymer-based films are traditionally formed either by casting or extrusion. In the casting method, biopolymer solutions are poured onto a mould, followed by gelation and drying. Cold-set biopolymers (e.g., gelatin, alginate, carrageenan, and gellan gum) are poured onto a mould as a hot sol typically in the presence of a polysaccharide-sensitive ion (e.g., alginate and calcium) to induce gelation as temperatures are cooled down. In contrast, heat-set biopolymers

Table 2.1 Functional properties of biodegradable edible films.

Functionalities	Definition and importance	Detection methods
Moisture sorption	Hydrophilic nature of edible films results in the absorption of water and hydrates under high RH environment, which decreases structural integrity, resistance to moisture transport, and mechanical strength.[1]	Swelling index (SI) = $(W_2–W_1)/W_1×100$ (W_1 = the weight of original film, W_2 = the weight of the film which is immersed into distilled water for 24 hours).[2] Moisture sorption isotherm (MSI): measures the water content of the films that are stored at different equilibrium RHs under a specific temperature.[3]
Water vapor permeability (WVP)	WVP is defined as water vapor transmission rate per unit area which is induced by the vapor pressure difference between the food and its surrounding environment under specified temperature and RH. Because many deteriorative chemical and enzymatic reactions, microbial growth, and textural properties of certain foods are governed by water activity and water content of foods, WVP of film is very important. [4]	WVP is determined by "cup method" (ASTM E96-93) based on the gravimetric technique. The film is sealed on a cup partially filled with the solution and stored in an air desiccators under controlled RH and temperature, and measuring the weight gain or loss of the film over time.[5, 6, 7, 8]
Optical property	Optical property of edible films refers to the transparency of films which depends on the formulation and fabrication procedures of films. It is crucial important for attractive ability of foods.[2]	A spectrophotometer is usually used to determine film opacity, and the adsorption spectrum is measured over a wavelength range of 400-800 nm.[9]

Table 2.1 Functional properties of biodegradable edible films (continued).

Functionalities	Definition and importance	Detection methods
Gas permeability	Gas permeability, the gas (O_2, CO_2, and aroma) transmission rate, is measured by unit gas pressure between food and the environment under specified temperature and humidity conditions. Due to lipid oxidation, enzymatic reaction, and respiration of postharvest fruits and vegetables, controlling O_2 and CO_2 permeabilities are very important; and aroma permeability is significant for the maintenance of flavor and aroma of foods.[4]	O_2 and CO_2 permeability is determined by ASTM D3985-02 method: the film is placed between two chambers under specific RH and constant temperature, one contains O_2 and CO_2 which can pass through the film and goes into another chamber which contains N_2, and O_2 permeability is measured by O_2 sensor, and CO_2 is determined by gas chromatography.[10]
Mechanical properties	The mechanical properties of film which include tensile and puncture strengths which reflect the ability of the film to resist external physical stress. Tensile strength (TS), tensile elongation (TE), elastic modulus (E) puncture strength (PS), and puncture deformation (PD) are mainly concerned. The improvement of mechanical properties of films can increase yield, facilitate handling, and protect foods from mechanical damage during food transportation.[4]	Tensile testing is performed using a TA.XT2 Texture Analyzer according to the ASTM D882-91 to determine TS, TE, and E; puncture testing is also measured using a TA.XT2 Texture Analyzer to determine PS and PD; and both of tests are operated in a specified RH (usually 54% RH at room temperature is applied).[2, 11]

References: adapted from: [1]Greener & Fennema (1989); [2]Gontard et al. (1992); [3]Gontard et al. (1993); [4]Janjarasskul & Krochta (2010); [5]Banker et al. (1966); [6]Kamper & Fennema (1985); [7]Kester & Fennema (1986); [8]Martin-Polo & Voilley (1990); [9]Gontard et al. (1994); [10]ASTM D3985-02 (2002); [11]ASTM D882-91 (1991).

Table 2.2 General overview of various biodegradable edible film materials.

Film materials	Formation mechanism	Advantages	Disadvantages	Examples
Polysaccharide – based films	Coacervation process disrupts interactions among long-chain polymer segments, and new intermolecular hydrophilic and hydrogen bonding are formed upon evaporation of the solvent to create a film matrix.[1, 2]	Materials are abundant, low cost, and easy to handle. Good gas and lipid barrier properties. Used in controlled delivery applications. Moderately good mechanical properties at low relative humidity (RH).[1, 2, 3]	Mechanical strength is weak at high RH. Poor moisture barrier property, highly water soluble.[1, 2]	Cellulose derivatives, starch, pectin, alginate, carrageenan, chitosan.[1, 2]
Protein – based films	Involves protein denaturation by heat and pH of solvents, followed by dehydration and cross linking. Casting or extrusion method are commonly used.[1, 2]	Used in controlled delivery applications. Good barrier property to against gases, aromas, and lipids.[4, 5]	The film is brittle and susceptible to cracking. High water vapor permeability.[1, 2]	Wheat gluten, corn zein, soy protein isolate, collagen and gelatin, milk proteins.[1, 2]
Lipid – based films	Involves dipping a supporting mold into a molten lipid, followed by cooling.[1, 2]	Low water vapor permeability. Induces a sheen on the surface of food product.[2, 6]	Poor mechanical properties, including being non-self-supporting. Waxy taste/texture. Greasy surface. Potential rancidity. Fragile and not cohesive.[1, 2]	Glycerol esters, waxes, resin, surfactants (C_{16} – C_{18} fatty acids and fatty alcohols).[1, 2]

Table 2.2 General overview of various biodegradable edible film materials (continued).

Film materials	Formation mechanism	Advantages	Disadvantages	Examples
Composite films	Bi-layer film: Films are formed in two stages. 1st stage: the layer of polysaccharide or protein is casted and dried, 2nd stage: the lipid layer is combined.[7, 8]	Better water vapor barrier efficiency, and moderately good mechanical properties at low RH.[2, 4, 9]	The bi-layer structure has a tendency to crack and/or delaminate. Complicate processing steps.[2, 7]	Combining lipid compounds with a hydrocolloid-based structural matrix.[1, 2]
	Emulsion-film: Films are derived using a stable lipid-protein (or polysaccharide) emulsion. The lipid is dispersed in an hydrophilic phase (protein or polysaccharide) to form an emulsion.[5, 8]	Good mechanical strength. Simple process for manufacture, being applied on food at room temperature, adhesive.[1, 2, 9]	Less efficient due to non-homogeneous distribution. Stability issues relating to lipid melting temperature and solvent volatilization lead to loss in structure. Poor control over moisture transfer.[1, 2]	

References: adapted from: [1]Vargas et al. (2008); [2]Janjarasskul & Krochta (2010); [3]Baldwin et al. (1995); [4]Debeaufort & Voilley (1995); [5]Shellhammer & Krochta (1997); [6]Greener & Fennema (1989); [7]Krochta (1997); [8]Perez-Gago & Krochta (2005); [9]Gontard et al. (1994).

Table 2.3 Plant protein-based edible films found in the literature.

Film type	Formulation	Processing	Tests	Reference
Lentil protein	LPC (5%), Gly (50%)	Film forming solution (70ºC/20 min/pH 11.0); Setting conditions (25ºC/48h/50%RH)	Thickness, color, mechanical properties, WVP, TSM	Bamdad et al. (2006)
Faba bean protein	FPI (5%), Gly (40%, 50%, 60%)	Film forming solution (room temperature/pH 7.0, 9.0, and 12.0); Setting conditions (25ºC/48h/50%RH)	Thickness, color, mechanical properties, WVP, TSM, SEM analysis	Saremnezhad et al. (2011)
Soy protein	SPI (6%, 7%, 8%, 9%), Gly (40%, 50%, 60%, 70%)	Film forming solution (70ºC/20 min/pH 7.0); Setting conditions (25ºC/48h/30%RH)	Thickness, DSC, WVP	Kokoszka et al. (2010)
Soy protein	SPI (8.33%), Gly (50%), genipin (0%, 0.1%, 1%, 2.5%, 5%, 7.5%, 10%)	Film forming solution (70ºC/2 h/pH 9.0); Setting conditions (25ºC/48h/50%RH)	Thickness, opacity, TSM, mechanical properties, WVP, SEM analysis	Gonzalez et al. (2011)
Soy protein	SPI (5%), Gly (60%), Sor (60%), MTGase (4 units)	Film forming solution (70ºC/20 min/pH 8.0); Setting conditions (25ºC/48h/50%RH)	Thickness, tensile test, WVP, TSM, transparency, SEM analysis	Tang et al. (2005)
Pea protein	PPI (10%), Gly (20%, 30%, 40%, 50%)	Film forming solution (90ºC/25 min)	Tensile test, WVP, TSM	Choi & Han (2001)
Rapeseed protein	RP (4%), Sor/Sucrose (1.5%/0.5%, 1.5%/1.5%, 2.0%/0.5%, 2.0%/1.0%)	Film forming solution (room temperature); Setting conditions (25ºC/48h/50%RH)	Tensile test, WVP, SEM analysis	Jang et al., (2011)
Sunflower protein	ISFP (10%), Gly (50%), PEGs (40%, 50%, 60%)	Film forming solution (150ºC/3 min); Setting conditions (25ºC/48h/60%RH)	Mechanical properties, WVP	Orliac et al., (2003)

Abbreviations: lentil protein concentrate (LPC); faba bean protein isolate (FPI); soy protein isolate (SPI); pea protein isolate (PPI); rapeseed protein (RP); sunflower protein isolate (ISFP); glycerol (Gly); sorbitol (Sor); polyethylene glycols (PEGs); microbial transglutaminase (MTGase); relative humidity (RH); water vapor permeability (WVP), total soluble matter (TSM); scanning electron microscopy (SEM); differential scanning calorimetry (DSC)

(e.g., soy protein, whey protein, and oval albumin) are poured onto the mould at room temperature. As temperatures are raised, proteins can be denatured and aggregated with neighboring proteins via hydrophobic interactions and covalent linkages to induce 'particulate-type' or 'fibrous-type' gel networks (Kester & Fennema, 1986; Debeaufort et al., 1998; Janjarasskul & Krochta, 2010). Proteins are quite sensitive to changes in temperature. Within the film formation processes, proteins may be disaggregated, dissociated and denatured by heating, which then promotes protein-protein aggregation as protein molecules re-align and associate with each other (Redl et al., 1999). The addition of cross linking agents and plasticizers are carefully balanced to ensure improve both film strength and flexibility once set (Pommet et al., 2003). Choi & Han (2002) prepared pea protein isolate (PPI)-based films through heating the film forming solution at 90 ºC for 5, 10, 20, 30, 40, and 50 min. The authors found that the heat treatment significantly improved the tensile strength and elongation of films, where the tensile strength and elongation of heat-denatured (20 min) PPI films were 7 and 13 times higher than non-denatured PPI films, respectively. Degassing of the film forming solution is essential to reduce the chance of air bubbles, as the material dries (Yang et al., 2010). During drying, the aqueous solvent is removed leading to significant increases in biopolymer concentration, aggregation and chain entanglement to form a self-supporting film. The film is then conditioned to a desired relative humidity before testing.

In the extrusion method, thermally-induced phase transition (e.g., in soy protein) (Cunningham et al., 2000), glass transition (e.g., in gelatin) (Park et al., 2008), and gelatinization characteristics (e.g., in starches) (Pushpadass et al., 2009) are important considerations in the film production (Janjarasskul & Krochta, 2010). Processing typically involves heating the biopolymers above their glass transition temperature (T_g) under low moisture conditions, and eventually leading to a uniform melt which can be easily shaped into films/packages using heat and pressure upon cooling, or thermal compression or injection molding. The thermal extrusion is more cost effective with higher output than the casting method for making films, and the formation, aggregation, and cross linking structures in the film are highly dependent on processing temperature, drying rate, and screw speed in the thermal extrusion (Rhim & Ng, 2007; Hernandez-Izquierdo & Krochta, 2008; Hernandez-Izquierdo et al., 2008). Many carbohydrates and proteins, such as sodium alginate (Liu et al., 2006), corn zein (Wang & Padua, 2003), and soy protein (Cunningham et al., 2000) exhibit potential thermoplastic behaviors for the film

formation by thermal extrusion.

Lipid-based films are typically prepared by: a) melting the lipid material, followed by re-solidification; b) solubilizing the lipid material within an organic solvent, followed by evaporation; or c) creating an oil-in-water emulsion, followed by evaporation of the aqueous phase (Greener & Fennema, 1989; Gontard & Guilbert, 1994; Janjarasskul & Krochta, 2010).

Composite materials involving two (or more) biopolymers (e.g., proteins and polysaccharides) are also used in film production (Janjarasskul & Krochta, 2010), where gelation is induced via a process known as complex coacervation whereby two biopolymers with opposite net charges interact via electrostatic attractive forces within a narrow pH range (Janjarasskul & Krochta, 2010). This range typically extends from pHs > pK_a of the reactive site on the polysaccharide backbone (e.g., alginate, -COO- pK_a of 1.88) and pHs < pI (isoelectric point of a protein, e.g., whey protein pI 4.6), where the polysaccharide and protein assumes a net negative and positive charge, respectively. In contrast, composite films involving proteins/polysaccharides and lipids can be produced using a layer-by-layer stacking technique to form a laminate-type film or through the creation of an emulsion-based gel (and then film matrix) whereby lipid-droplets are dispersed within the biopolymer matrix (Perez-Gago & Krochta, 2005).

2.2 Plasticizers

Plasticizers are typically added to biopolymer-based films to overcome brittleness issues to make films more malleable and allow the films to be easily removed from the moulds (Sothornvit & Krochta, 2005; Janjarasskul & Krochta, 2010). Plasticizers are small poly alcohol (-OH) molecules added to the film forming solution to disrupt intermolecular interactions between chains and to replace polymer-polymer interactions with polymer-plasticizer interactions (via hydrogen bonding); resulting in a heterogeneous distribution of junction zones and the increase of chain mobility within the film matrix to make the film more flexible (Hettiarachchy & Eswaranandam, 2005; Sothornvit & Krochta, 2005; Janjarasskul & Krochta, 2010). In general, plasticizers situate themselves into the polymeric network to disrupt the hydrogen bonding between neighboring polymers, reduce the intermolecular attractive forces, and increase the intermolecular space, thereby, allowing for improved flexibility, extensibility, and toughness of the films (Hettiarachchy & Eswaranandam, 2005; Sothornvit & Krochta, 2005; Janjarasskul & Krochta, 2010). On the other hand, since plasticizers lessen the attractive

forces and increase the free volume in the film matrix, the diffusion coefficient for gases and water vapors are increased (Banker, 1966; Guilbert, 1986; Hettiarachchy & Eswaranandam, 2005).

Plasticizers used in the production of biodegradable edible films can be divided into water soluble (e.g., glycerol) and insoluble (e.g., saturated fatty acids) plasticizers (Siepmann et al., 1998). Hydrophilic plasticizers dissolve in the aqueous medium to provide more space between polymer chains when they are added into film forming solution. Theoretically, due to the hydrophilic nature, water soluble plasticizers result in an increase of water diffusion within film structure. In contrast, water insoluble plasticizers lead to a decrease in the water uptake of films. However, phase separation or formation of discontinuity zones within the film structure may result from the addition of water insoluble plasticizers to further decrease film flexibility and water vapor barrier property. Therefore, the optimum stirring for the film forming solution is critical for the application of water insoluble plasticizers (Bodmeier & Paeratakul, 1997). Moreover, in polymer science, plasticizers can be defined as internal (e.g., sorbitol and sucrose) or external (e.g., linseed oil and castor oil) plasticizers depending on the interactions between plasticizers and polymers. In brief, external plasticizers are low volatile substances which cannot chemically react with polymers through primary bonds and will be eventually lost by evaporation. Internal plasticizers have bulky structures to co-polymerize or react with original polymers to inhibit polymer-polymer interactions from occurring, therefore, films will be soften as evidenced by reduced elastic modulus values (Frados, 1976; Sothornvit & Krochta, 2005).

Both type and amount of plasticizers affect the interactions between biopolymers and plasticizers. For instance, film extensibility and flexibility can be increased and the film strength can be decreased as the concentrations of plasticizers are raised. Plasticizers with lower molecular weight and higher surface charge can easily insert into the film matrix to increase the plasticizing effect (Sothornvit & Krochta, 2001; Hettiarachchy & Eswaranandam, 2005). The compatibility of plasticizers with biopolymers is related to the plasticizers' size, shape, space between oxygen atoms, as well as their water-binding abilities. Plasticizers must be readily soluble in the film forming solution and miscible with all polymers present. Polyols (e.g., glycerol, sorbitol, and polyethylene glycols), mono-, di-, or oligosaccharides (e.g., glucose, fructose-glucose syrups, and sucrose), lipids and their derivatives (e.g., phospholipids and surfactants) are the most commonly used plasticizers in the films (Sothornvit & Krochta, 2005).

2.3 Cross linking agents

In order to withstand the external stress and moisture environment that would occur during processing and handling of products, biodegradable edible films should have proper strength, flexibility, and barrier properties to maintain the integrity of products (Yang & Paulson, 2000b). Therefore, many researchers have been focused on improving film properties by means of cross linking using physical, chemical and enzymatic treatments.

Ultraviolet and γ-irradiation can be used to produce cross links in protein-based films; however, the efficiency at improving film properties is highly dependent upon the properties of the protein being used, especially the amino acid composition and molecular structure/conformation. For instance, the tensile strength of soy protein films was increased by 65%, whereas the tensile elongation for the same film decreased by 31% with the application of UV irradiation (0.0104 J/cm^2). In this case, the aromatic amino groups (e.g., tyrosine and phenylalanine) in soy protein participated within the cross linking reaction. In contrast, wheat gluten and pea protein films were not affected by γ-irradiation (Tomihata et al., 1992; Gennadios et al., 1998; Micard et al., 2000). Protein cross links can also form upon heating the film forming solution, following a similar mechanism as heat set gelation of globular proteins. During this process, proteins are completely or partially unraveled to expose hydrophobic moieties that were previously buried within the interior of the protein, followed by protein interactions via hydrophobic interactions and possibly disulfide bridging (Damodaran, 2008).

Depending on the material and film strength, cross linking agents may be added to the material being casted (e.g., transglutaminase + protein/chitosan; genipin + gelatin/chitosan) (Yajima et al., 2010; Porta et al., 2011). Chemical cross linking agents, typically containing aldehyde groups, can react with the amino groups of lysine residues to form bridges between protein chains (Song et al., 2011). Glutaraldehyde is the most commonly used chemical cross linking agent. However, due to its high toxicity, its application in biodegradable edible films has been limited from the consideration of safety issues. Recently, a new natural cross linker, genipin have been used in the production of films. It is about 10,000 times less cytotoxic than glutaraldehyde (Yuan et al., 2007; Song & Zhang, 2009). Gonzalez et al. (2011) evaluated the properties of soy protein isolate (SPI)-based films with the addition of varying levels of genipin. The authors reported that mechanical and water vapor barrier properties were significantly improved by adding only a small amount (< 2.5% w/w genipin relative to the SPI) to the film

forming solutions.

In contrast, enzymatic cross-linking agents are more popular and beneficial in the production of films (Song et al., 2011). Enzymatic cross linking agents (e.g., peroxidase and transglutaminase) can produce polymers with high molecular weight by catalyzing covalent cross linking reactions between proteins (Song et al., 2011). Because of the reduction of tensile strength and elongation by the application of peroxidase in the films (Michon et al., 1999), transglutaminase is more commonly used in film production, such as in the case of soy protein films (Tang & Jiang, 2007) and wheat gluten films (Tang et al., 2005). Transglutaminase catalyzes the acyl transfer of the γ-carboxyamide group of glutamine into the ε-amino group of lysine to release ammonia and introduces the ε-(γ-glutamyl)-lysine cross links in the protein molecules (Folk, 1980).

In general, cross linking agents in the film act to reduce film solubility, improve film strength, reduce swelling and decrease gas/water vapor permeability by increasing macromolecular interactions within the film. For instance, Porta and co-workers (2011) reported the application of $CaCl_2$ to cross link casein-based films enhanced protein-protein interactions, and led to a 31% reduction in the film thickness and decreased solubility.

2.4 Other film additives

2.4.1 Emulsifiers

Emulsifiers may be added, especially to composite films involving both biopolymer and lipid materials. Emulsifiers are surface active molecules with both polar and non-polar ends that act to modify the lipid-water interface (e.g., reduced interfacial tension) to make the two immiscible phases more stable (Krochta, 2002; Janjarasskul & Krochta, 2010). They can be incorporated into the film formulations to improve the dispersion of lipid particles and reduce interfacial tension of the solution to achieve sufficient surface wettability and adhesion of films (Krochta, 2002). Rhim and co-workers (1999) observed that soy protein isolate-based films became thicker, stronger, and less susceptible to shrinkage with the addition of fatty acids (e.g., lauric acid, palmitic acid, stearic acid, and oleic acid). Some common emulsifiers used in film production include: acetylated monoglyceride, lecithin, polysorbate 60, and glycerol monopalmitate. Furthermore, proteins themselves have some emulsifying properties owing to their amphiphilic nature (Janjarasskul & Krochta, 2010).

2.4.2 Waxes

In order to improve the barrier properties associated with biopolymer-based films, waxes are commonly used as additives in the film formulations. Wax is a type of lipid with a long-chain fatty acid and tends to be solid at room temperature, and has high hydrophobicity (Kester & Fennema, 1986). Natural waxes (e.g., carnauba wax, candelilla wax, and rice bran wax) can be extracted from plants and seeds by nonpolar solvents, therefore, waxes cannot be solubilized into the aqueous solutions (Baldwin, 2007; Song et al., 2011). Because of the hydrophobic long-chain ester and free fatty alcohol in the molecular structure, waxes behave as desirable additives to improve the water vapor permeability of films. The water vapor permeability and total soluble matter of soy protein isolate-based films were gradually decreased with an increase of sorghum wax from 5% to 20% (w/w of protein) (Kim et al., 2002). However, the addition of waxes in the film formulation can decrease the mechanical strength and make the film become fragile, because waxes have poor ability to form covalent bonds with biopolymers in the film structure (Janjarasskul & Krochta, 2010). Moreover, there are some other disadvantages associated with the application of waxes in the films, such as greasy appearance and waxy taste and texture (Janjarasskul & Krochta, 2010). Beeswax, petroleum wax, carnauba wax, and candelilla wax are commonly used with biopolymers in the film formulations (Baldwin, 2007).

2.5 Choice of materials

2.5.1 Canola protein isolate

Canola (*Brassicaceae spp.*) is primarily grown for its oil content to be used for cooking and biodiesel purposes (Wu & Muir, 2008). Once the oil is pressed, the remaining meal (high in protein and fiber) is typically sold as low price feed products (Uruakpa & Arntfield, 2005). Canola meal is relatively high in protein content (up to 50% protein on a dry basis) (Uppstrom, 1995), has a well-balanced amino acid profile (Table 2.4) (Chabanon et al., 2007), and has good technologically functional properties (Aluko & McIntosh, 2001). In order to improve the viability of the canola industry, proteins are now being extracted from the meal as a value-added by-product for both food and non-food applications. However, despite this, protein products haven’t gained any traction as new food ingredients until recently. A few companies (e.g., BioExx Specialty Protein (Toronto, ON, Canada) and Burcon NutraSciences (Vancouver, BC,

Table 2.4 Amino acid composition of napin and cruciferin (expressed as mass percent) (Chabanon et al., 2007).

Amino acid	Napin	Cruciferin
Aspartic acid + asparagine	5.1	9.5
Glutamic acid + glutamine	30.4	20.2
Serine	4.1	4.4
Histidine	4.7	5.1
Glycine	1.9	1.7
Threonine	4.4	4.7
Alanine	3.4	3.5
Arginine	8.6	9.8
Tyrosine	3.7	4.5
Cysteine	0.1	0.0
Valine	4.3	3.3
Methionine	0.5	1.2
Phenylalanine	3.7	5.7
Isoleucine	4.3	5.3
Leucine	8.5	9.1
Lysine	6.2	4.7
Proline	6.4	6.8
Tryptophan	n.d.	n.d.

Abbreviation: not determined (n.d.)

Canada)) are looking to start moving canola protein ingredients into the marketplace. As such, researches on value-added opportunities for the canola protein sector, such as edible packaging, is important for long term industry sustainability.

Canola proteins are dominated by a salt-soluble globulin protein (cruciferin) and a water-soluble albumin protein (napin), constituting approximately 60% and 20% of the total protein, respectively (Hoglund et al., 1992). Cruciferin (12S; S is a Svedberg unit; molecular weight of 300 kDa; pI of 7.25) is a hexameric protein comprised of six subunits, each being composed of a heavy α-chain with 254 to 296 amino acids and a light β-chain with 189 to 191 amino acid residues linked by one disulfide bond (Wanasundara, 2011). It is considered as a

neutral protein. Denaturation of cruciferin was found to occur at 91 ºC as evidenced by a major endothermic peak during a differential scanning calorimetry (DSC) study (Wu & Muir, 2008). In contrast, napin (2S; molecular weight of 12.5-15 kDa; pI of 11) is a much smaller protein comprised of a 4.5 kDa polypeptide linked together with a 10 kDa polypeptide by two disulfide bonds. Napin is very hydrophilic and soluble at neutral pH. Denaturation of napin was found to occur at 110 ºC by DSC investigation (Wu & Muir, 2008; Wanasundara, 2011). Napin is characterized by strong alkalinity that is due to its high level of basic amino acid (e.g., histidine, lysine, and arginine), which leads to its very basic pI (Schmidt et al., 2004).

2.5.2 Plasticizers

To improve the flexibility and to overcome brittleness of biodegradable edible films, plasticizers (e.g., glycerol, sorbitol, and polyethylene glycol 400 (PEG-400)) are typically added to soften the structure (Gennadios et al., 1996; Cao et al., 2009; Mikkonen et al., 2009). The effectiveness is dependent on the composition, size, and shape of the plasticizer used (Sothornvit & Krochta, 2001). Glycerol ($C_3H_8O_3$; molecular weight of 92.09 g/mol) (Figure 2.1) is the most commonly used plasticizer due to its low molecular weight and hydrophilic nature (Redl et al., 1999; Cunningham et al., 2000). Glycerol disperses throughout the biopolymer network via hydrogen bonding to disrupt intermolecular interactions between chains and replace polymer-polymer interactions with polymer-plasticizer interactions. This results in a heterogeneous distribution of junction zones within the matrix, which increases free volume and allows chains to be more mobile within the films, which ultimately improves film flexibility (Sothornvit & Krochta, 2005; Janjarasskul & Krochta, 2010).

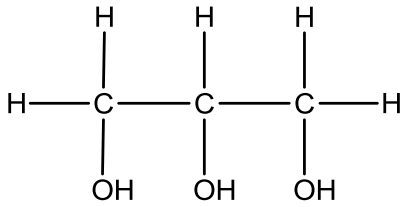


Figure 2.1 The chemical structure of glycerol.

Sorbitol ($C_6H_{14}O_6$; molecular weight of 182.17 g/mol) (Figure 2.2) is a polyhydric sugar alcohol, and is also widely used in biodegradable edible films due to its high water solubility,

polarity and compatibility with the film matrix (Barreto et al., 2003). Sorbitol had been extensively used as a plasticizer in the production of films, such as gelatin-based films (Cao et al., 2009), polyvinyl alcohol/rambutan skin waste flour films (Ooi et al., 2012), and egg albumen films (Gennadios et al., 1996).

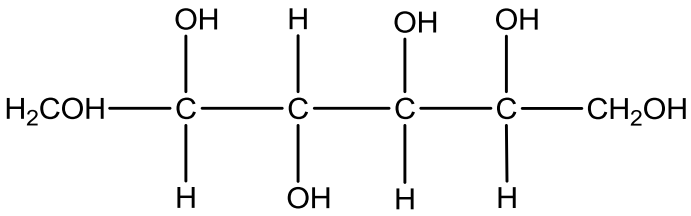


Figure 2.2 The chemical structure of sorbitol.

Due to the low toxicity and good solubility, polyethylene glycol (PEG) (Figure 2.3), which is a non-ionic polymer comprised of ethylene oxide units of varying molecular weights (300, 400, 600, 800, 1500, 4000, 10000, and 20000 g/mol), is another commonly used plasticizer. PEG molecules have good water solubility and hygroscopic properties due to the presence of two hydroxyl groups (-OH) at the ends of each chain (Annunziata et al., 2002; Cao et al., 2009). The plasticizer efficiency partially depends on the hydrogen bonding ability of plasticizer to replace the polymer-polymer interactions by polymer-plasticizer interactions. Theoretically, hydrogen bonding ability of a plasticizer is determined by the number of hydroxyl groups, solubility, and polarity. Turhan and co-workers (2001) found that with the increase of molecular weight of PEG, its polarity, solubility, and ability for hydrogen bonding interactions with polymers decreased. Therefore, PEG of high molecular weight might be undesirable to form sufficient hydrogen bonds with polymers in the film matrix. In contrast, PEG of low molecular weight has a large number of hydroxyl groups per mole (e.g., PEG-400) to be easily inserted into the polymer matrix and exhibits a plasticizing effect (Cao et al., 2009).

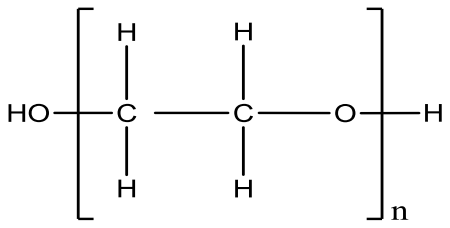


Figure 2.3 The chemical structure of polyethylene glycol (PEG).

2.5.3 Genipin

Genipin (Figure 2.4), a natural chemical cross linking agent, is obtained from the enzymatic hydrolysis of *Genipa* extracted from *Gardenia Jasminoides Ellis* fruit with β-glucosidease (Fujikawa et al., 1987). Recently, genipin is being tested in film applications for biomedical purposes as an alternative to glutaraldehyde because of its low cytotoxicity and proliferative capacity for cells (Yuan et al., 2007; Song & Zhang, 2009). Once cross linked, a dark blue pigment develops (Touyama et al., 1994). Genipin was used in the traditional Chinese medicine to treat type-2 diabetes (Zhang et al., 2006). The cross linking reactions between genipin and the primary amines (mainly lysine) within the protein are favored under acid or neutral conditions, in which the nucleophilic attack occurs on the olefinic carbon atoms of genipin to form an intermediate aldehyde group and open the dihydropyran ring, then, followed by the attack on the resulting aldehyde group by amine group (Figure 2.5, Scheme 1). The other half of cross linking is believed to form via an S_N2 nucleophilic substitution reaction, in which the ester group on genipin is replaced by a secondary amide group to release a methanol molecule (Figure 2.5, Scheme 2) (Muzzarelli, 2009; Gonzalez et al., 2011). Genipin reacts with the primary amines (mainly lysine) within the protein and forms both inter- and intramolecular cross links. Genipin has been used previously to fix chitosan-based films to control swelling and improve tensile strength (Jin et al., 2004; Liu et al., 2012). Recently, genipin has also fixed films derived from silk fibroin and sericin (Motta et al., 2011) and soy protein (Gonzalez et al., 2011). Due to the low cytotoxicity and applications in pharmaceuticals, genipin was used in the present study to investigate its effects on the properties of canola protein isolate (CPI)-based films.

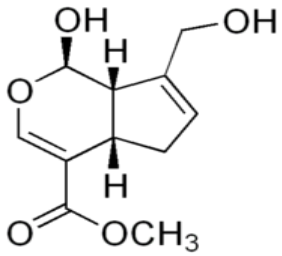
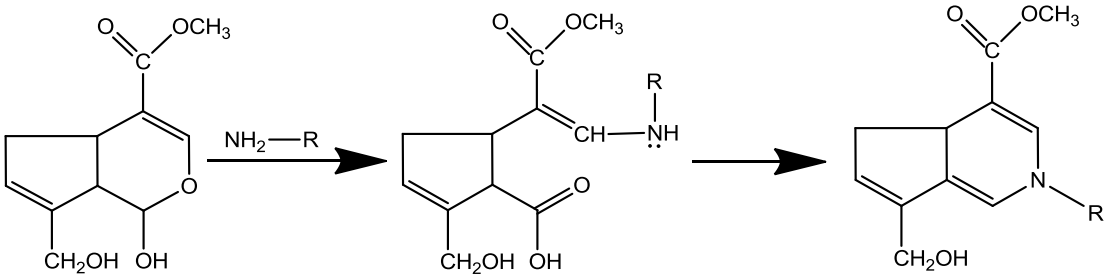
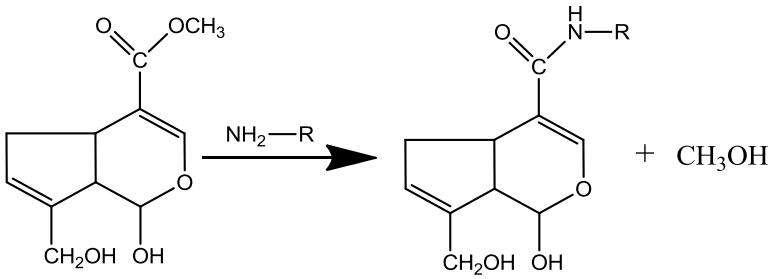


Figure 2.4 The chemical structure of genipin (Fujikawa et al., 1987).



Scheme 1



Scheme 2

Figure 2.5 Cross linking reactions between genipin and primary amines.

2.6 Summary

In conclusion, canola protein isolate (CPI)-based films will be prepared by glycerol, sorbitol, and PEG-400 with and without genipin in the present research. In order to better understanding the role of plasticizers in the development of biodegradable edible films, mechanical, optical, and water vapor barrier properties of films will be investigated as a function of both plasticizer-type and plasticizer content. The addition of genipin in the film will be aimed to improve mechanical strength and water vapor barrier properties of CPI films.

3. EFFECT OF PROTEIN AND GLYCEROL CONCENTRATION ON THE MECHANICAL, OPTICAL AND WATER VAPOR BARRIER PROPERTIES OF CANOLA PROTEIN ISOLATE-BASED EDIBLE FILMS

3.1 Abstract

Biodegradable edible films prepared using proteins are both economically and environmentally important to the food packaging industry relative to traditional petroleum-derived synthetic materials. In the present study, the mechanical and water vapor barrier properties of casted canola protein isolate (CPI) edible films were investigated as a function of protein (5.0% and 7.5%) and glycerol (30%, 35%, 40%, 45%, and 50% (w/w of CPI)) contents. Specifically, tensile strength (TS) and elongation (TE), elastic modulus (E), puncture strength (PS) and deformation (PD), opacity, and water vapor permeability (WVP) were measured. Results indicated that TS, PS, and E decreased, while TE and PD values increased as glycerol concentration increased for both 5.0% and 7.5% CPI films. Furthermore, TS, PS, and E values were found to increase at higher protein concentrations within the CPI films, whereas PD values decreased. TE was found to be similar for both CPI protein levels. CPI films became more transparent with increasing of glycerol concentration and decreasing of CPI concentration. WVP value was also found to increase with increasing glycerol and protein contents. Overall, results indicated that CPI films were less brittle, more malleable and transparent, and had greater water vapor permeability at higher glycerol levels. However, as protein level increased, CPI films were more brittle, less malleable and more opaque, and also had increased water vapor permeability.

3.2 Introduction

Due to the increased concerns on over consuming synthetic petroleum-based packaging in the food industry over the past decade, researchers are focusing on the development of biodegradable edible packaging as an alternative to synthetic petroleum-based packaging. Traditional petroleum-derived synthetic materials used in food packaging do not only cause the environmental pollution, but also create tremendous demands in landfills (Gontard et al., 1993;

Kowalczyk & Baraniak, 2011). As such, researchers have been investigating natural biopolymer-based materials (e.g., protein-, polysaccharide- and lipid-based) which are both economically and environmentally important in terms of food packaging to develop biodegradable edible films as alternatives to synthetic petroleum-based packaging. In addition, because of the material selected for the production of biodegradable edible films, films may also have the added advantage of being edible and/or being used as a controlled delivery platform to improve product quality and safety (e.g., release of bioactive compounds, such as antioxidants), or to extend shelf-life (e.g., release of antimicrobial compounds) (Han & Gennadios, 2005). Biopolymer-based films are originated from naturally renewable resources, such as proteins (e.g., gelatin, whey, soy and corn zein), polysaccharides (e.g., alginate, carrageenan, chitosan, and pectin), and lipids (e.g., beeswax). Protein- and polysaccharide-based materials tend to form films with excellent mechanical properties and gas barrier properties, but poor moisture control (Kester & Fennema, 1989; Baldwin et al., 1995; Vargas et al., 2008; Janjarasskul & Krochta, 2010). In contrast, lipid-based films tend to have excellent moisture barrier properties, but have poor mechanical and gas barrier properties (Greener & Fennema, 1989; Janjarasskul & Krochta, 2010). The formation of edible films using proteins from plant sources has been limited, but may be advantageous to those from animal sources because of their low cost, and consumer perceived safety concerns (i.e., prions) or dietary restrictions over consuming animal-derived products (Uppstrom, 1995; Gennadios, 2002). Films have been prepared previously using proteins from plant sources, such as, soy (Cho & Rhee, 2004), sunflower (Orliac et al., 2002), lentil (Bamdad et al., 2006), faba bean (Saremnezhad et al., 2011), pea (Choi & Han, 2001; Kowalczyk & Baraniak, 2011) and rapeseed (Jang et al., 2011).

Canola proteins have received some interests over the past few decades in terms of their functional attributes (Aluko & McIntosh, 2001, Yoshie-Stark et al., 2008), however, despite this, protein products haven’t gained any traction as new food ingredients until recently. A few companies (e.g., BioExx Specialty Proteins (Toronto, ON, Canada) and Burcon NutraSciences (Vancouver, BC, Canada)) are looking to start moving canola protein ingredients into the marketplace. Canola (*Brassicaceae spp*.) is primarily grown for its oil content used for cooking and biodiesel purposes (Wu & Muir, 2008). Once the oil is pressed, the remaining meal (high in protein and fiber) is typically used in feed applications (Canola Council of Canada, 1990; Uruakpa & Arntfield, 2005). In order to improve the viability of the canola industry, proteins are

now being extracted from the meal as a value-added by-product for both food and non-food applications. Canola proteins are dominated by a salt-soluble globulin protein (cruciferin, 11S, molecular weight of 300 kDa) and a water-soluble albumin protein (napin, 2S, molecular weight of 12.5-15 kDa), constituting ~60% and ~20% of the total proteins, respectively (Wanasundara, 2011).

The formation of films generally involves some levels of protein denaturation, followed by surface dehydration either at room temperature or within a drying oven. Protein denaturation is required in order to induce unfolding to give a more open structure and to expose a greater number of reactive sites which partake in various intermolecular interactions (e.g., disulfide bridging, hydrogen and ionic bonding, and hydrophobic interactions) to form the films (Krochta, 1997). Plasticizers, such as glycerol (or another small poly alcohol (-OH) molecule), are often added to protein-based films to overcome brittleness issues; making films more malleable by disrupting hydrogen bonds between neighboring proteins to reduce intermolecular attractive forces (Guilbert, 1986; Kester & Fennema, 1986). Glycerol also acts to create a heterogeneous distribution of junction zones within the protein matrix to make the film more flexible (Sothornvit & Krochta, 2005; Janjarasskul & Krochta, 2010).

Generally, protein-based films should be able to maintain integrity and withstand external stress from processing, handling, and storage; meaning they should have adequate mechanical strength and extensibility, to be competitive with traditional petroleum-derived packaging (Yang & Paulson, 2000b). Films should also be able to provide some moisture barrier properties. The overall aim of the present study is to investigate the effect of protein and glycerol concentrations on the mechanical, optical and water vapor barrier properties of an edible film casted using canola protein isolate (CPI).

3.3 Material and methods

3.3.1 Materials

Canola seeds (B. napus /variety VI-500) were kindly donated by Viterra (Saskatoon, SK, Canada) for this study. All chemicals used in this study were reagent grade, and purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Milli-Q water was produced from a Millipore Milli-Q^{TM} water purification system (Millipore Corporation, Milford, MA, USA).

3.3.2 Preparation of a canola protein isolate

Canola seeds (stored at 4^{o}C in a sealed container prior to use) were initially screened based on size using first a #8 (2.63 mm) Tyler mesh filter (Tyler, Mentor, OH, USA) and then a #12 (1.70 mm) filter. The screened seed was frozen at -40^{o}C overnight, and then were cracked using a stone mill (Morehouse-Cowles stone mill, Chino, CA, USA). The seed coat and cotyledons were then separated using an air classifier (Agriculex Inc., Guelph, ON, Canada). The cotyledons oil was removed up to ~13% mechanically using a continuous screw expeller (Komet, Type CA59 C; IBG Monforts Oekotec GmbH & Co., Mönchengladbach, Germany), which was operated at a speed of 59 rpm using a 3.50 mm choke. The residual oil in the meal was removed by hexane extraction (x3) at a 1:3 meal to hexane ratio for 8 h. The meal was then air-dried for an additional 8 h to allow for residual hexane to evaporate. CPI was prepared from defatted canola meal according to the method described by Folawiyo & Apenten (1996) and Klassen and other co-workers (2011). In brief, 100 g defatted canola meal was dissolved in 1000 g 0.05 M Tris-HCl buffer containing 0.1M NaCl (pH = 7.0) at room temperature (21-23^{o}C) for 2 h under constant mechanical stirring at 500 rpm (IKAMAG RET-G, Janke & Kunkel GMBH & Co. KG, IKA-Labortechnik, Germany). The solution was then centrifuged (Sorvall RC Plus Superspeed Centrifuge, Thermo Fisher Scientific, Asheville NC, USA) at 3000 × *g* for 1 h to collect the supernatant. This was then filtered using # 1 Whatman filter paper (Whatman International Ltd., Maidstone, England), dialyzed (Spectro/Por tubing, 6-8 kDa cut off, Spectrum Medical Industries, Inc, USA) at 4 ºC for 72 h with frequent changes of Milli-Q water (Millipore Corporation, MA, USA) to remove the salt, and then freeze-dried (Labconco Corporation, Kansas City, Missouri 64132) at a temperature difference of 35 ºC for 24 h to yield the CPI powder for later use.

The crude protein composition of CPI powder was determined using the Association of Official Analytical Chemists Method 920.87 (AOAC, 1995). The CPI produced was found to be comprised of 90.45% protein (%N x 6.25). CPI concentration used in this study reflected the protein content rather than powder weight.

3.3.3 Preparation of canola protein isolate films

Film forming solutions were prepared by slowly dissolving CPI (5.0% and 7.5% protein w/w) in Milli-Q water under constant mechanical stirring at 500 rpm (IKAMAG RET-G, Janke

& Kunkel GMBH & Co. KG, IKA-Labortechnik, Germany), adjusted to pH 3.0 using 1 M HCl, and then allowed to stir for 1 h at room temperature (21-23^{o}C). In the preliminary experiments, adjusting the CPI film forming solution to pH 8.0 by using 0.1 NaOH was tried to solubilize CPI, however, since CPI in the present study was dominated by cruciferin (pI of 7.25), CPI cannot be solubilized at pH 8.0. Glycerol was then added at 30, 35, 40, 45, and 50% (w/w of CPI) into the film forming solutions, and then allowed to stir (500 rpm) for an additional 10 min. Table 3.1 gives the contents of each film formulation tested. The film forming solutions were then degassed for 10 min within an ultrasonic bath at a frequency of 40 kHz (Branson Ultrasonic Cleaner, Model 2510R-DTH, USA) at room temperature (21-23^{o}C). Afterwards, the film forming solutions were heated to 50 ºC under stirring at 500 rpm for 5 min, and then casted onto a polytetrafluoroethylene (PTFE) mould (10 cm length; 10 cm width; 0.10 mm depth). During preliminary experiments, heating the film forming solution to 70 ºC was also tried to make the CPI films, however, since the concentration of protein was really high, once the temperature reached to 70 ºC, the film forming solution formed a gel. As such, heating to only 50^{o}C seemed more optimal. Excess film forming solutions were removed using a straight edge. CPI films were formed after drying overnight at room temperature (21-23^{o}C). The thickness of film was controlled by the standard depth of the PTFE mould, the time of drying process, and the amount of film forming solution (~15 ml) poured on the mould. Films were then removed from the mould, and conditioned to 54% relative humidity (using a saturated magnesium nitrate solution) within a desiccator at room temperature (21-23^{o}C) for 2 d. All films were prepared in triplicate. The addition of glycerol was decided based on the preparation of pure CPI films. Because there were intra- and intermolecular interactions between side chains of partially denatured CPI, molecular mobility in the film structure was restricted which leading to very brittle pure CPI film. Therefore, glycerol was added to decrease the interactions between protein chains and improve the malleability of CPI films (Zhang et al., 2001; Kokoszka et al., 2010). Glycerol levels within the prepared films were restricted to the range between 30 and 50%, since at levels <30%, films became too brittle and experience cracking during the drying process, whereas at levels >50%, films were too soft and sticky to be removed from the moulds after drying (data not shown). CPI levels within the prepared films were restricted between 5.0% and 7.5%, since at levels <5.0%, films were too thin to be removed from the mould as a full piece of film, whereas at levels >7.5%, films with 50% glycerol experienced cracking during the drying process (data not

shown).

Table 3.1 Composition of CPI film forming solutions prior to film casting.

Film	CPI (g)	CPI (% db)	Gly (g)	Gly (%/CPI)	Water (g)	Thickness (mm)
5.0% CPI, 30% Gly	5.0	77	1.50	30	93.50	0.06
5.0% CPI, 35% Gly	5.0	74	1.75	35	93.25	0.07
5.0% CPI, 40% Gly	5.0	71	2.00	40	93.00	0.07
5.0% CPI, 45% Gly	5.0	69	2.25	45	92.75	0.07
5.0% CPI, 50% Gly	5.0	67	2.50	50	92.50	0.07
7.5% CPI, 30% Gly	7.5	77	2.25	30	90.25	0.12
7.5% CPI, 35% Gly	7.5	74	2.63	35	89.87	0.10
7.5% CPI, 40% Gly	7.5	71	3.00	40	89.50	0.13
7.5% CPI, 45% Gly	7.5	69	3.38	45	89.12	0.13
7.5% CPI, 50% Gly	7.5	67	3.75	50	88.75	0.13

3.3.4 Film thickness

Film thickness was measured by using a digital micrometer (Model 62379-531, Control Company, U.S.A.) having a precision of 0.01 mm. Ten thickness measurements were taken on each triplicate film prepared.

3.3.5 Mechanical properties

Puncture strength and deformation

Both puncture strength (PS, N) and deformation (PD, mm) of the film were determined using a Texture Analyzer (Texture Technologies Corp., New York) as described by Gontard and co-workers (1992). Each film was mounted on a 65.6 mm diameter puncture mould and placed under a smooth edged cylindrical probe (4 mm diameter), the probe then moved through the film at a cross-head speed of 1 mm/s. The force-deformation curve data were collected by a microcomputer. PS was the maximum force (N) which was loaded on the film to puncture the specimen. PD was expressed as the length changes at the rupture point of film.

Tensile strength, tensile elongation and elastic modulus

Tensile strength (TS, MPa), tensile elongation (TE, %), and elastic modulus (E, Pa) of the film were determined using a Texture Analyzer with a load cell of 25 kg (Texture Technologies Corp., New York) on film strips (8 × 2.5 cm) which were pre-conditioned at 54% relative humidity under room temperature based on the ASTM D882-91 (1991). The film strips were placed between grips, and set up the initial grip separation to 40 mm and cross-head speed to 5 mm/s. The stress-strain curve data were collected by a microcomputer. TS was calculated by dividing the maximum load of the film strip by the area of cross-section of that strip (width of the strip (2.5 cm) × thickness of the strip); TE was calculated as a percentage of the length change of the film strip at the breakpoint of the film; E was expressed as the slope of the trend line on the stress-strain curve. Three measurements were taken on each triplicate film prepared.

3.3.6 Opacity

Film opacity was determined by using a spectrophotometer (Genesys 10uv, Thermo Fisher Scientific) as described by Gontard and co-workers (1994). The pre-conditioned films were cut into small strips (4.5 x 0.9 cm) and placed on the inside wall of the plastic cuvette (1 cm path length). The absorption spectrum will be measured over a wavelength range of 400–800 nm. The area under the absorbance-wavelength curve was determined as the film opacity with the unit of A.nm. All measurements were performed in triplicate, for each type of film.

3.3.7 Water vapor permeability

Water vapor permeability (WVP) of the CPI films was determined using the "cup method" modified from the gravimetric technique of ASTM E96-93 (1993). For this study, PVC (polyvinyl chloride) cups (Figure 3.1) were prepared to the following dimensions: outer cup height (2.65 cm), outer cup radius (2.50 cm), inner cup height (2.00 cm) and inner cup radius (2.25 cm). Films were placed on the top of the cup, then held in place by a lid (with an open centre of same dimensions as the inner cup radius) tightened by six screws. The open surface area of the film was 15.90 cm^2. Within the cup, 10 mL of saturated $Mg(NO_3)_2$ solution (54% relative humidity) was added. The entire cup (with $Mg(NO_3)_2$ solution plus film) was then placed within a desiccator containing $CaSO_4$ desiccant (0% relative humidity) at room temperature. The water transferred through the film was determined from the weight loss of the system (cup plus

Figure 3.1 An image of the cup used in measurement of water vapor permeability (WVP).

$Mg(NO_3)_2$ solution) over a 5 h duration at 30 min intervals, and weighed to the nearest 0.1 mg using an analytical balance (CPA224S, Sartorius, U.S.A.). Preliminary experiments (not shown) showed that a steady state of weight loss was reached after 5 h. WVP of the film was calculated using the WVP Correction Method which was described as the following formulae (Gennadios et al., 1994).

$$WVP = \frac{WVTR_m \times L}{P_{w1} - P_{w2}} \quad [3.1]$$

$$P_{w1} = P_T - (P_T - P_{w0})\exp\left(N_w h / cD\right) \quad [3.2]$$

$$N_w = \left(6.43 \times 10^{-11}\right) \times WVTR_m \quad [3.3]$$

where $WVTR_m$ (water vapor transmission rate, g/m^2s) was calculated by dividing the slope by the open area of the cup (15.90 cm^2); and L was the thickness of the film (mm). P_{w1} was water vapor partial pressure at the film inner surface (kPa), P_{w2} was the water vapor partial pressure at film outer surface (kPa). Since the cup was placed in the desiccator containing $CaSO_4$ desiccant (0% relative humidity), the P_{w2} was 0 kPa. P_T was the total atmospheric pressure (101.3 kPa); P_{w0} was the partial pressure of water vapor at the air of the surface of the $Mg(NO_3)_2$ solution which was 1.34267 kPa; N_w ($g.mol/s.cm^2$) was the measured value of $WVTR_m$; h was the stagnant air gap height between the film and the surface of $Mg(NO_3)_2$ solution; c was the total

molar concentration of the air and water vapor (4.15×10^{-5} g.mol/cm^3); D was the diffusivity of water vapor through the air at 25 ºC (0.25375 cm^2/s). All measurements were performed on triplicate films.

3.3.8 Statistical analyses

All experiments were performed on triplicate films and reported as the mean ± one standard deviation. A two-way analysis of variance (ANOVA) was used to measure statistical differences in thickness, mechanical properties (PS, PD, TS, TE and E), and opacity, and WVP of CPI films among the various treatments (e.g., effect of glycerol and CPI concentrations).

3.4 Results and discussion

3.4.1 Mechanical properties

Film strength

The effect of glycerol and protein concentrations on strength (PS, TS and E) of CPI films were examined and given in Figures 3.2A, C and E. An analysis of variance of PS data indicated that glycerol ($p<0.001$) and protein concentration ($p<0.001$), along with their interaction ($p<0.01$) were all significant. Overall, PS data were found to be higher at the 7.5% CPI films than the 5.0% CPI films, and declined as glycerol level increased from 30% to 50% (Figure 3.2A). However, the decline occurred at different rates depending on the protein concentration. This rate was slightly less at the 5.0% CPI films where PS value decreased from ~2.29 N to ~0.89 N as the glycerol content increased from 30% to 50%, respectively (Figure 3.2A). In contrast, PS value declined from ~3.87 N to ~2.05 N as glycerol levels increased at the 7.5% CPI films (Figure 3.2A). An analysis of variance on TS data indicated that both glycerol ($p<0.001$) and protein ($p<0.001$) concentrations were highly significant, however the interaction term was not significant ($p>0.05$). Overall, TS decreased with increasing glycerol content where TS declined from ~4.31 MPa for films with 30% glycerol to ~1.19 MPa with 50% glycerol present in 5.0% CPI films (Figure 3.2C). TS also was found to increase with increasing protein concentration from ~1.19 MPa to ~2.33 MPa when CPI concentration increased from 5.0% to 7.5% in films with 50% glycerol, respectively (Figure 3.2C). The lack of significant interaction suggested that the decline in TS with increasing glycerol content followed a similar trend at both protein concentrations. An analysis of variance of E data indicated that glycerol ($p<0.001$) and

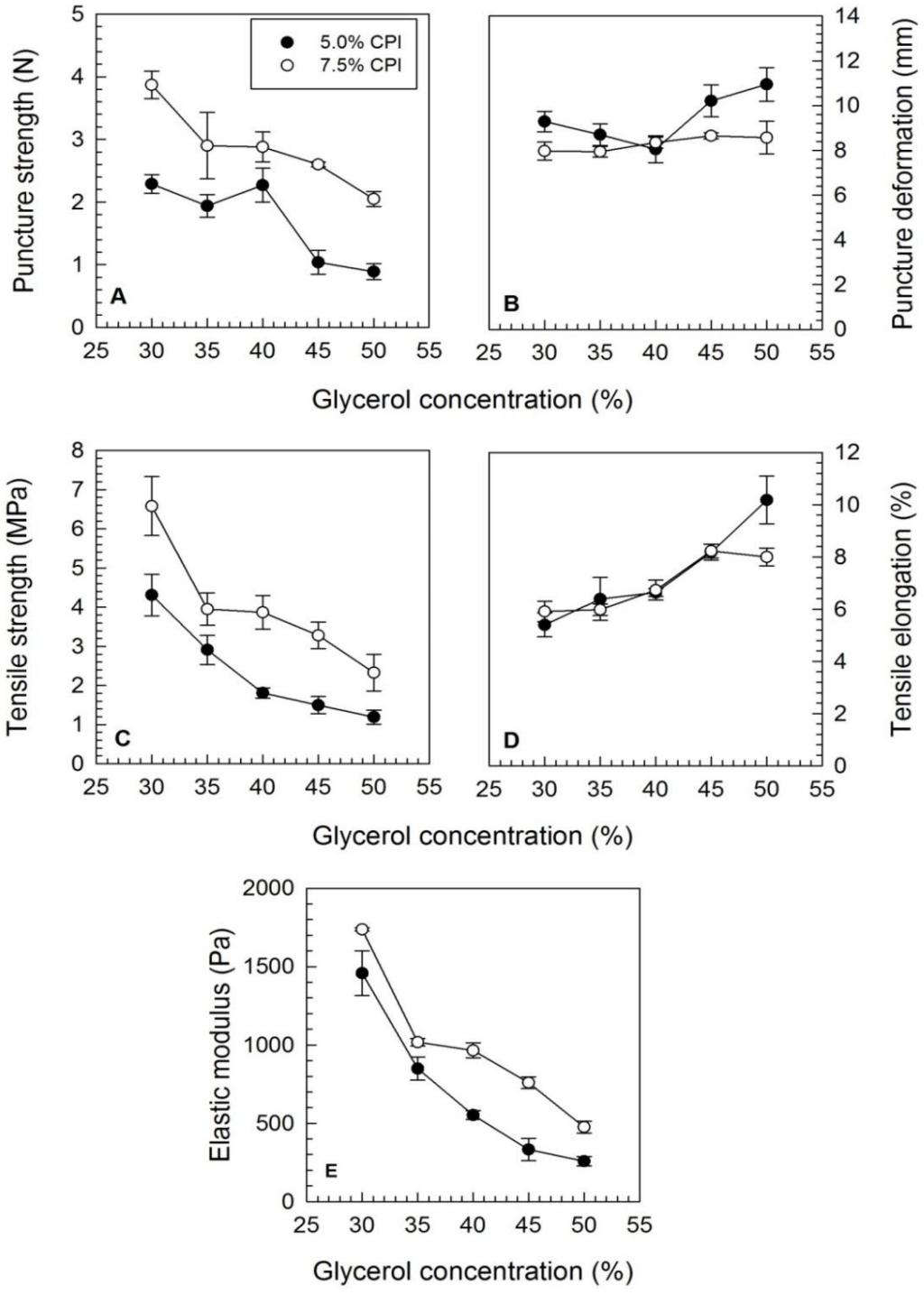


Figure 3.2 Puncture strength (A) and deformation (B), tensile strength (C) and elongation (D), and elastic modulus (E) of 5.0% and 7.5% (w/w) canola protein isolate (CPI) films as a function of glycerol concentration.

protein concentrations ($p<0.001$), along with their interaction ($p<0.01$) were all significant. Overall, E was greater for the 7.5% CPI films than at the 5.0% CPI films, and declined with increasing glycerol concentration. At the 5% CPI level, E data declined from ~1,458 Pa at 30% glycerol to ~258 Pa at 50% glycerol in a curvilinear decline with reduced rates between 40 and 50% glycerol (Figure 3.2E). In contrast, at the 7.5% CPI level, the decline was more consistent from ~1,737 Pa at 30% glycerol to ~476 Pa at 50% glycerol (Figure 3.2E).

Overall, the strength of CPI films was thought to increase due to a rise in intermolecular CPI interactions within the film matrix as protein levels increased. Since CPI can contribute to the noncovalent interactions (e.g., hydrogen bonding, hydrophobic interactions), the film structure was strengthened by the higher CPI concentration (Cao et al., 2007). Furthermore, heating to 50^{o}C under acidic conditions (pH 3.0) was proposed to induce some unfolding and then subsequent hydrophobic interactions between CPI aggregates and sulfhydryl exchange reactions between cysteine moieties (Folawiyo & Apenten, 1996). As the film forming solution was cooled down, the CPI film was proposed to be strengthen due to an increase in hydrogen bonding within the system (Fukshum & Vanburen, 1970). Film strength in the present study was also attributed to differences in film thickness, because film thickness greatly affects the film structure through the effect on the drying kinetics of the film forming solution. In fact, the thickness mainly depends on the solvent evaporation rate and the protein denaturation to affect cross links in film network organization (Debeaufort & Voilley, 1995). An analysis of variance of film thickness indicated that only protein concentration ($p<0.001$) was significant, and glycerol and their interaction ($p>0.05$) were not. Overall, films were ~0.12 mm thick at the 7.5% CPI level and ~0.07 mm thick at the 5.0% CPI level, regardless of the glycerol content (Table 3.1, p. 27). Jang et al. (2011) also reported a similar rise in thickness of rapeseed protein films from ~47.4 µm to ~71.6 µm as protein levels increased from 2% to 5%, respectively. On the whey protein isolate-based films, although the increased glycerol concentration slightly increased film thickness, it didn't significantly affect the film thickness (Gounga et al., 2007). Furthermore, 7.5% CPI films had higher CPI concentration by area unit, which could enhance the intermolecular interactions and lead to the formation of film matrix with higher cohesion (Cuq et al., 1996). This was demonstrated by Sobral (1999) on gelatin based films, in which PS value of films increased from 2.5 N to 30 N as the film thickness increased from 0.02 to 0.14 mm.

The film strengths reported in the present study using CPI was comparable to other plant

protein films (Table 3.2). Rhim and co-workers (1998) reported a soy protein film with 50% glycerol prepared at a 5.0% protein concentration had TS of ~6.34 MPa. Prepared under the same set of conditions, except at a lower soy protein concentration, Cho & Rhee (2004) reported 4.0% soy protein film had TS of ~3.20 MPa. This trend in TS data with decreasing protein concentration was similar to that of the present study. The increased film strength at higher protein level is presumed to reflect greater biopolymer ordering within the film. Puncture strength values for CPI films with 50% glycerol (~0.9 N or ~2.0 N for the 5.0% and 7.5% CPI level, respectively) also were within the similar range with 5.0% lentil protein-based films prepared with 50% glycerol (~1.6 N) (Bamdad et al., 2006) and the plastic sandwich wrap (~3.2 N) (Table 3.2). Liu et al. (2004) reported greater denaturation in protein-based films results in a more compact 3-diminsional microstructure with greater strength than those with less. According to Folawiyo & Apenten (1996), the film forming solution (5% CPI/50% Gly) was heated to 50^{o}C under acid condition (pH 3.0) for 5 min presumably allowing for partial denaturation of CPI; to give films with TS of ~1.19 MPa. In contrast, soy protein-based films (~6.34 MPa) (Rhim et al., 1998) and lentil protein-based films (~4.2 MPa) (Bamdad et al., 2006) were prepared through heating film forming solution to 70 °C for 20 min (at comparable protein (5%) and glycerol (50%) concentrations) gave films with higher TS values than CPI films (Table 3.2), because of greater protein denaturation within the film matrix. Theoretically, protein denaturation can increase intra- and intermolecular cross links to tighten the film structure, so, further greatly affects the properties of edible films through protein-protein interactions, and polymer morphology (Choi & Han, 2002). Choi & Han (2002) indicated that although 5 min heat treatment at 90 ºC was long enough to produce strong pea protein isolate (PPI) films, PPI films produced from the 20 min heat treatment were much stronger, due to the greater molecular rearrangement occurred during heating process. Although mechanical strength of 7.5% CPI films (PS of ~2.05 N) was considerably lower than the plastic sandwich wrap (PS of ~3.18 N) (Table 3.2), CPI films still could be considered as an acceptable packaging to replace synthetic petroleum-based packaging under moderate mechanical applications, such as separated packaging in the large box.

Table 3.2 Mechanical properties and water vapor permeability of various plant protein films found in the literature.

Film type	Formulation	Processing	TS (MPa)	TE (%)	PS (N)	WVP ($g.mm/m^2.h.kPa$)	Thickness (mm)
Soy protein[a]	5% SPI, 50% Gly	Film forming sol'n (70°C/20 min/pH 10.0); Setting conditions (25°C/48 h/50% RH)	6.34 ± 0.02	65.90 ± 25.30	-	5.40 ± 0.07	0.08 ± 2.50
Lentil protein[b]	5% LPC, 50% Gly	Film forming sol'n (70°C/20 min/pH 11.0); Setting conditions (25°C/48 h/50% RH)	4.24 ± 1.26	58.22 ± 12.88	1.55 ± 0.20	0.10 ± 0.00	0.15 ± 0.00
Pea protein[c]	10% PPI, 50% Gly	Film forming sol'n (90°C/25 min)	0.69 ± 0.07	92.00 ± 21.50	-	7.42 ± 0.69	5.83 ± 0.85
Sunflower protein[d]	10% ISFP, 50% Gly	Film forming sol'n (155°C/2 min); Setting conditions (25°C/48 h/60% RH)	2.80	37.60	-	-	-
Canola protein*	5% CPI, 50% Gly	Film forming sol'n (50°C/5 min/pH 3.0); Setting conditions (21-23°C/48 h/54% RH)	1.19 ± 0.18	10.18 ± 0.91	0.89 ± 0.13	1.20 ± 0.17	0.07 ± 0.01
Canola protein*	7.5% CPI, 50% Gly	Film forming sol'n (50°C/5 min/pH 3.0); Setting conditions (21-23°C/48 h/54% RH)	2.33 ± 0.47	8.00 ± 0.34	2.05 ± 0.12	1.50 ± 0.05	0.12 ± 0.01
Plastic sandwich wrap	-	-	-	-	3.18 ± 0.12	0.03 ± 0.00	-

References: [a]Rhim et al. (1998), [b]Bamdad et al. (2006), [c]Choi & Han (2001), [d]Orliac et al. (2002), *present study

Abbreviations: soy protein isolate (SPI); lentil protein concentrate (LPC); pea protein isolate (PPI); sunflower protein isolate (ISFP); glycerol (Gly); tensile strength (TS) and elongation (TE); puncture strength (PS); water vapor permeability (WVP); and relative humidity (RH)

The decline in film strength in the present study with increasing glycerol concentration is presumed due to its plasticizing effect. Glycerol disrupts the order of CPI-CPI aggregates within the film matrix, results in a more heterogeneous spatial distribution of junction zones to increase free volume within the film matrix to improve the polymeric chains mobility (Donhowe & Fennema, 1992; Sothornvit & Krochta, 2005; Janjarasskul & Krochta, 2010). Glycerol also displaces some stabilizing hydrogen bonding between water molecules and the CPI, by interacting themselves (-OH groups) with the CPI through hydrogen bonding (Gontard et al., 1993). Some researchers reported that glycerol decreased hydrogen bonding to further increase free volume between protein molecules in pea protein and peanut protein films (Choi & Han, 2001; Liu et al., 2004), thus promoted an increase of deformation capacity of film structure to reduce the film mechanical resistance (Donhowe & Fennema, 1992). As a consequence, films were weaker and more flexible as levels of glycerol increased. Slight differences seen in the rate of decline in PS and E values with increasing glycerol concentration between both CPI levels is thought to be associated with the distribution of glycerol molecules. It is proposed that 7.5% CPI film has a more tightly ordered matrix resulting in a more heterogeneous distribution of glycerol molecules. In contrast, at the 5.0% CPI level, the less ordered film matrix allowed CPI to re-orient to accommodate the presence of glycerol. It could be summarized that glycerol has less ability to restrict the interaction between polymer chains under bulky protein content in the film matrix. Changes to molecular dynamics of CPI as a function of CPI and glycerol concentrations within the film matrix were also reflective in the PD and TE (Figures 3.2B and D) data, where slight differences in trends were seen, despite the overall rise in film flexibility with increasing glycerol content. Choi & Han (2001) reported a similar trend for TS data as a function of glycerol concentration, where TS decreased from ~4.9 MPa to ~0.7 MPa as the glycerol concentration increased from 20% to 50%.

Film deformability

The effect of glycerol and protein concentration on flexibility (PD and TE) of CPI films were examined and given in Figures 3.2B and D. An analysis of variance of PD data indicated that both glycerol ($p<0.001$) and protein ($p<0.001$) concentrations, and their interaction ($p<0.01$) to be significant. Overall, PD decreased from ~10.95 mm to ~8.57 mm as the protein concentration increased from 5.0% to 7.5% in films with 50% glycerol, respectively (Figure

3.2B). However the effect of increasing glycerol content was different depending on the protein concentration. At the 5.0% CPI level, PD initially declined from ~9.29 mm to ~8.05 mm between 30% and 40% glycerol, and then increased from ~8.05 mm to ~10.95 mm between 40% and 50% glycerol level (Figure 3.2B). In contrast, the effect of glycerol at the 7.5% CPI level was less significant, increasing in a slow linear manner from ~7.97 mm to ~8.57 mm between 30% and 50% glycerol, respectively (Figure 3.2B). An analysis of variance of TE data found that both glycerol ($p<0.001$) and protein concentrations ($p<0.05$), along with their interaction ($p<0.001$) were significant. Overall, TE was slightly greater in 5.0% CPI films than in 7.5% CPI films, and increased as the glycerol level was increased. However the rate of increase was different depending on the protein concentration. For instance, at the 5.0% CPI level, TE data increased linearly from ~5.4% at 30% glycerol to ~10.2% at 50% glycerol (Figure 3.2D). In contrast, at the 7.5% CPI level, TE increased slowly between 30% and 40% glycerol from ~5.9% to ~6.7%, respectively, then jumped to ~8.2% at the 45% glycerol before reaching a plateau (Figure 3.2D).

Comparison of TE data for CPI films with those reported for other plant protein-based films indicated significantly lower values, probably due to the low pH (pH 3.0) used to prepare the CPI film forming solution. Gennadios and co-workers (1993) found that soy protein films could be formed at both alkaline (pH 7.0 to 11.0) and acidic conditions (pH 1.0 to 3.0), where significantly higher TE values were reported under the former conditions (132.6%-187.3%) than the latter (34.2%-35.6%). The authors presumed that this was caused by poor protein dispersion near to its isoelectric point (pI 4.5). Moreover, the heating time could be an additional reason, since the hydroxyl groups of glycerol can replace protein-protein interactions in denatured protein by developing protein-glycerol hydrogen bonds to increase the chain mobility during the film formation, and finally leads to the increase of flexibility of films (Gontard et al., 1993). However, in the present study, CPI film forming solution was only heated for 5 min which was much shorter than 20 min for other films. This theory was demonstrated by Choi & Han (2002) on PPI films, in which the films produced from 20 min heat treatment had 2.0 to 3.5 times higher TE value than films produced from 5 min heat treatment.

3.4.2 Film opacity

Transparency (low opacity) of the prepared film is an important factor to consider in terms of designing food packages (depending on the product). In the present study, the color of films was slightly yellowish, and 7.5% CPI films were darker and more yellow than 5.0% CPI films. Film opacity of CPI films as a function of protein and glycerol concentrations was shown in Figure 3.3. An analysis of variance indicated that the opacity of the films was affected by both glycerol ($p<0.001$) and protein ($p<0.001$) concentration, along with their interaction ($p<0.05$). Overall, opacity of the film with 50% glycerol prepared at the 7.5% CPI level was greater than at the 5.0% CPI level, where values decreased from ~83.5 A.nm to ~76.4 A.nm, respectively (Figure 3.3). However, the rate of decline in opacity differed depending on the glycerol concentration. For instance, at the 5.0% CPI level, opacity value declined linearly from ~96.0 A.nm to ~76.4 A.nm as glycerol level increased from 30% to 50%, respectively (Figure 3.3). In contrast, at the 7.5% CPI level, opacity value was relatively constant between 30% and 40% glycerol contents, with opacity values ranging between ~94.8 and ~96.6 A.nm, respectively, then declined sharply to ~84.5 A.nm at the 45% glycerol level where it started to remain constantly (Figure 3.3). Gontard et al. (1994) reported that opacity value of films declined with increasing glycerol content, due to the transparent nature and increased dispersion of glycerol within the film matrix. Differences in trends between the two protein concentrations in the present study are thought to reflect the distribution of glycerol molecules within the film, where it is proposed that at the higher CPI level, a more heterogeneous distribution of glycerol occurs. The higher opacity value is presumed to be associated with the higher total solid contents in the 7.5% CPI film, the more tightly packed CPI network and the greater thickness relative to the 5.0% CPI film.

3.4.3 Water vapor barrier property

Water vapor permeability (WVP) of CPI films as a function of glycerol and protein concentrations was investigated, and shown in Figure 3.4. An analysis of variance of WVP data found that both glycerol ($p<0.001$) and protein ($p<0.001$) concentrations were significant, however their interaction ($p>0.05$) was not significant. Overall, WVP was found to increase from ~1.20 to ~1.50 $g \cdot mm/m^2 \cdot h \cdot kPa$ as CPI concentration was raised from 5.0% to 7.5% in films with 50% glycerol, respectively (Figure 3.4). Additionally, WVP also increased from ~0.94 to ~1.50 $g.mm/m^2.h.Pa$ as glycerol concentrations increased from 30% to 50% in a slightly curvilinear

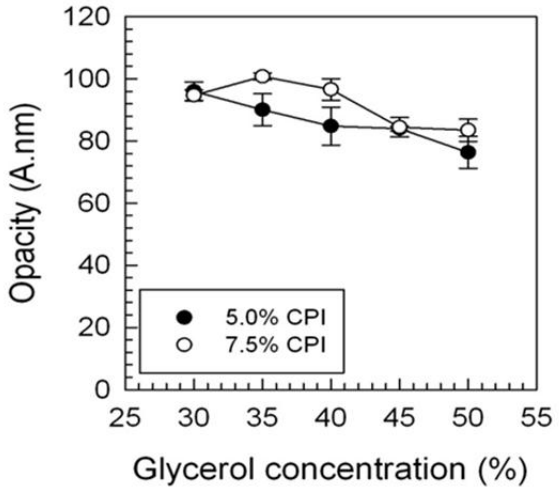


Figure 3.3 Opacity of 5.0% and 7.5% (w/w) canola protein isolate (CPI) films as a function of glycerol concentration.

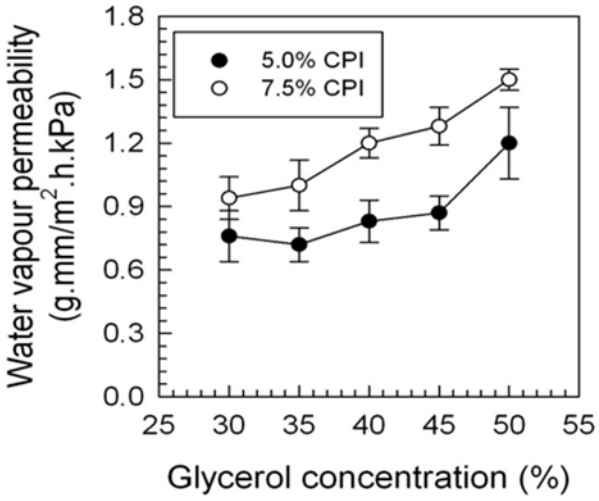


Figure 3.4 Water vapor permeability (WVP) of 5.0% and 7.5% (w/w) canola protein isolate (CPI) films as a function of glycerol concentration.

trend in 7.5% CPI films (Figure 3.4). Choi & Han (2001) also reported similar results on 10% pea protein films where the WVP increased from ~4.30 to ~7.42 $g \cdot mm/m^2 \cdot h \cdot kPa$ as the glycerol concentration increased from 20% to 50%. The rise in WVP with increasing glycerol concentration is proposed to reflect an increase in inter-chain spacing and biopolymer mobility within the film matrix, and a decrease in internal hydrogen bonding within the film structure, leading to increased diffusion of water molecules (Gontard et al., 1993; Yang & Paulson, 2000a; Gounga et al., 2007). The rise in WVP with the increase of glycerol content may also be related to the rise in water absorption caused by the addition of hydrophilic material in the films; enabling greater water diffusion through the matrix (Kamper & Fennema, 1984). Karbowiak and co-workers (2006) found that the moisture content of biopolymer films which mainly control the water molecular mobility is greatly affected by the plasticizer. Therefore, since the plasticizing action of glycerol is favorable to adsorption and absorption of water molecules in the film structure (Coupland et al., 2000); the increased glycerol content can substantially increase WVP of films.

WVP of CPI films was much lower in comparison with other plant proteins films, but it was higher than WVP of plastic sandwich wrap (Table 3.2, p. 34). This could be caused by a number of factors, such as film thickness, relative humidity (RH) for WVP measurement, and protein hydrophobicity. McHugh et al. (1993) stated that the thicker film had higher resistance to mass transfer across it, so, water vapor partial pressure at the film inner surface (P_{w1}) was increased to illustrate the much higher WVP of lentil protein film and pea protein film than CPI films (Table 3.2). In the present study, film solubility was also measured by the swelling index of film in which the film strip was dissolved into water for 24 h to measure the weight different of the film strip. However, due to the hydrophilic nature, CPI film trip was dissolved into water immediately (less than 2 min). It indicated that CPI films had weaker intramolecular interactions in the aqueous condition. Therefore, CPI films had much higher WVP than plastic sandwich wrap (Table 3.2, p. 34). Due to the higher solubility, CPI films could be appropriate for the application of hot water soluble pouches (Bamdad et al., 2006). In Table 3.2 (p. 34), the inner cup RH for WVP measurement on soy protein, lentil protein, and pea protein films was ~75% (Rhim et al., 1998; Choi & Han, 2001; Bamdad, et al., 2006), however, WVP of CPI films was measured when the inner cup RH was 54% which is mostly close to relative humidity at room temperature (21-23 ºC) in the environment, so, P_{w1} for CPI films was lower than P_{w1} for soy

protein, lentil protein, and pea protein films, which means soy protein, lentil protein, and pea protein films that are hydrophilic films exhibit higher WVP values, due to the water-film interaction (Banker et al., 1966). This theory was also demonstrated by Kokoszka et al. (2010) in soy protein-based films where the WVP of films at ~70% RH was much higher than the films at ~23% RH. In Table 3.2, it was found that CPI films had lower WVP than soy protein film. Hydrophobic amino acid profile in protein could be contributed to this result. Hydrophobic amino acids (leucine, proline, and alanine) account for ~19.40% of CPI (Chabanon et al., 2007), but ~15.64% of soy protein (Wang et al., 2008).

In the present study, WVP was also found to be greater for the films with 7.5% CPI content than with 5.0% CPI content. The trend is somewhat counterintuitive, more aggregated film structure with a denser protein matrix and larger pore size supposed to be formed with higher protein concentration (Gounga et al., 2007). Moreover, it was hypothesized that the higher amount of CPI allowed for a greater amount of CPI-water interactions than the lower amount of CPI, allowing for greater water mobility through the film matrix. It was demonstrated that WVP in rapeseed films increased from ~0.60 to ~0.88 $g \cdot mm/m^2 \cdot h \cdot kPa$ with the increase of rapeseed protein concentration from 2% to 5% (Jang et al., 2011). Film thickness was also greater at the 7.5% CPI level than the 5.0% CPI level, suggesting that water molecules would take a longer pathway to go through the films with higher amount of CPI, so, more hydrophilic film (7.5% CPI film) would be able to keep more water molecules within the film matrix. Since the time period (5 h) for WVP measurement on CPI films was same in this study, 7.5% CPI films had higher WVP values than 5.0% CPI films. In addition, McHugh and co-workers (1993) observed that films with greater thickness had increased resistance to moisture transfer, so, a stagnant air layer formed on the inner film surface to characterize as a higher water vapor partial pressure for WVP measurement. Therefore, 7.5% CPI films had higher WVP values than 5.0% CPI films.

3.5 Conclusions

The present study investigated the effect of glycerol and protein concentrations on the mechanical, optical and water vapor barrier properties of CPI films. In general, as the glycerol concentration was increased, films became weaker, more flexible and clearer. In contrast, as CPI concentration was raised, films became stronger, less flexible and more opaque. Water vapor barrier property also became poorer as both glycerol and CPI concentrations increased. This

study shows the potential of using CPI in the development of edible films/packaging.

3.6 Linkage

CPI films were prepared with different concentrations of glycerol. Although 5.0% CPI films with 50% glycerol had lower mechanical strength and poor water vapor barrier property, they had higher flexibility and transparency relative to other CPI films with glycerol. The focus of the second study of this research project was to investigate the effect of plasticizer-type on the mechanical, optical, and water vapor barrier properties of CPI films to better understand the role of plasticizer in the production of CPI films, and to further improve the mechanical resistance and moisture barrier property of films by the addition of genipin. In study two, films were prepared at 5.0% CPI and 50% glycerol based on preliminary experiments involves the addition of genipin. The effect of a cross linker was hypothesized to lead to more brittle films, so staring with the weakest film allowed for strength to be accessed without making the films more fragile.

4. EFFECT OF PLASTICIZER-TYPE AND GENIPIN ON THE MECHANICAL, OPTICAL, AND WATER VAPOR BARRIER PROPERTIES OF CANOLA PROTEIN ISOLATE-BASED EDIBLE FILMS

4.1 Abstract

The mechanical properties, opacity, and water vapor permeability of 5.0% (protein w/w) canola protein isolate (CPI) films were investigated in the presence and absence of 1% (w/w of CPI) genipin, and as a function of plasticizer-type (50% (w/w of CPI); glycerol, sorbitol, and polyethylene glycol 400). Findings indicated that tensile strength (TS), puncture strength (PS) and elastic modulus (E) values for CPI films prepared with sorbitol were the highest, followed by PEG-400 and then glycerol, whereas tensile elongation (TE) and puncture deformation (PD) values were greater for films prepared with glycerol, followed by PEG-400 and then sorbitol. In all cases, films prepared with genipin were stronger (greater TS, PS and E) and less flexible (lower TE and PD) than uncross linked films. Films also showed greater water vapor permeability (WVP) when prepared with glycerol, followed by PEG-400 and then sorbitol, however no differences were observed in the presence and absence of genipin. The results of present study suggested that CPI is a potential material for the development of edible films/packaging.

4.2 Introduction

Edible films developed from biodegradable materials (e.g., proteins, polysaccharides, and lipids) have attracted much attention by the food industry, as consumers' demands for alternatives to traditional petroleum-based packaging which negatively impacts the environment and landfills have been increased (Gontard et al., 1993; Kowalczyk & Baraniak, 2011). Biodegradable edible films prepared from proteins (e.g., gelatin, wheat gluten, and peanut protein), polysaccharides (e.g., chitosan, pectin, and starch), and lipids (e.g., beeswax and resin) provide mechanical and barrier properties, as well as can be formulated to act as a delivery system for bioactives (e.g., sodium alginate-gellan gum contaning N-acetylcysteine and glutathione (Rojas-Grau et al., 2007)) or antimicrobial compounds (e.g., hydroxyl propyl methyl cellulose-based film containing nisin

(Sebti & Coma, 2002)) to maintain product quality and extend shelf-life (Han & Gennadios, 2005). Typically, protein- and polysaccharide-based films tend to have good mechanical and gas barrier properties, but poor water vapor barrier property due to their hydrophilic nature (Janjarasskul & Krochta, 2010). In contrast, lipids-based films are poor at controlling gas diffusion and withstanding mechanical stresses, but good at controlling moisture migration due to their hydrophobic nature (Janjarasskul & Krochta, 2010). Because of perceived safety concerns (e.g., prion disease) and some dietary restrictions associated with using animal-derived proteins to prepare the films, plant proteins, such those from soy (Tang et al., 2005; Pruneda et al., 2008); sunflower (Orliac et al., 2003); faba bean (Saremnezhad et al., 2011); and rapeseed (Jang et al., 2011) represent as an excellent alternative.

Canola (*Brassicaceae spp.*) is primarily grown today for its polyunsaturated fatty acid rich oil, used for cooking and biodiesel purposes (Wu & Muir, 2008). A by-product arising from the oil industry is a protein- and fiber-rich canola meal that is underutilized in the marketplace, sold traditionally for use as a livestock feed. The protein content within the meal can be up to 50% on a dry weight basis and has a well-balanced amino acid profile (Uppstrom, 1995). The majority of these proteins are a salt-soluble globulin protein, known as cruciferin (11S; molecular weight ~300 kDa; ~60% of the total proteins) and a water-soluble albumin protein, known as napin (2S; molecular weight ~12.5-15 kDa; ~20% of the total proteins) (Wanasundara, 2011). Although the functional attributes of canola protein concentrates or isolates produced from the meal, such as protein solubility, emulsion stability, and foaming capacity, have been investigated (Aluko & McIntosh, 2001), their applications for food industry, such as for packaging, still need to be explored.

In an effort to tailor the mechanical and barrier properties of protein-based films, various factors have been previously explored including protein concentration (Jang et al., 2011), plasticizer concentration/type (Gennadios et al., 1996; Cao et al., 2009; Mikkonen et al., 2009), film forming conditions (i.e., pH, temperature and the presence of salts) (Kowalczyk & Baraniak, 2011; Saremnezhad et al., 2011); and the addition of cross linking agents (Tang et al., 2005; Tang & Jiang, 2007; Gonzalez et al., 2011). To improve the flexibility and to overcome brittleness of films, plasticizers (e.g., glycerol, sorbitol, and polyethylene glycol 400) are typically added to soften the structure (Gennadios et al., 1996; Cao et al., 2009; Mikkonen et al., 2009). The effectiveness is dependent on the composition, size, and shape of plasticizer used (Sothornvit &

Krochta, 2001).

Moreover, the formation of cross links by the addition of enzymatic or chemical fixatives has also been shown to influence film properties. For instance, genipin (GP), a natural chemical cross linking agent extracted from *Gardenia Jasminoides Ellis* fruit has showed some promise, as it can result in cross links of similar strength as glutaraldehyde but is 10,000 times less cytotoxic (Song & Zhang, 2009). GP reacts with the primary amines (mainly lysine) within the protein for the formation of both inter- and intramolecular cross links. Once reacted, a dark blue pigment develops (Touyama et al., 1994). Recently, genipin cross linking was used to fix films derived from chitosan (Jin et al., 2004), silk fibroin and sericin (Motta et al., 2011), and soy protein (Gonzalez et al., 2011).

The overall goal of the present research was to investigate the effect of plasticizer-type and GP on the mechanical, optical, and water vapor barrier properties of canola protein isolate (CPI) films. Enhanced utilization of canola proteins may increase their integration into the vegetable protein ingredient market.

4.3 Materials and methods

4.3.1 Materials

Canola seeds (B. napus /variety VI-500) were kindly donated by Viterra (Saskatoon, SK, Canada) for this study. All chemicals used in this study were reagent grade, and purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada) with the exception of GP (CAS Number: 6902-77-8, Challenge Bioproducts Co., Ltd, Taiwan). Milli-Q water was produced from a Millipore Milli-Q^{TM} water purification system (Millipore Corporation, Milford, MA, USA).

4.3.2 Preparation of a canola protein isolate

Canola seeds (stored at 4^{o}C in a sealed container prior to use) were screened based on size using first a #8 (2.63 mm) Tyler mesh filter (Tyler, Mentor, OH, USA) and then a #12 (1.7 mm). The screened seed was frozen at -40^{o}C overnight, and followed by the dehulling, so, the seeds were cracked by using a stone mill (Morehouse-Cowles stone mill, Chino, CA, USA). The seed coat was then removed from the cotyledons using an air classifier (Agriculex Inc., Guelph, ON, Canada). About 13% of cotyledons oil was pressed mechanically using a continuous screw expeller (Komet, Type CA59 C; IBG Monforts Oekotec GmbH & Co., Mönchengladbach,

Germany), which was operated at a speed of 59 rpm using a 3.5 mm choke. The residual oil in the meal was removed by hexane extraction (x3) at a 1:3 meal to hexane ratio for 8 h. The meal was then air-dried for an additional 8 h to allow for residual hexane to evaporate. CPI extraction from defatted canola meal was performed according to the method of Folawiyo & Apenten (1996) and Klassen and co-workers (2011). Defatted canola meal was extracted with 0.05 M Tris-HCl buffer, pH 7.0, containing 0.1M NaCl at at ratio of 1:10 (w:w, meal: solvent) with stirring for 2 h at room temperature (21-23^{o}C). The solution was centrifuged (Sorvall RC Plus Superspeed Centrifuge, Thermo Fisher Scientific, Asheville NC, USA) at 3000 × *g* at 4 ºC for 1 h to collect the supernatant, which was then filtered using # 1 Whatman filter paper (Whatman International Ltd., Maidstone, England), and dialyzed (Spectro/Por tubing, 6-8 kDa cut off, Spectrum Medical Industries, Inc, USA) at 4 ºC for 72 h with frequent changes of Milli-Q water (Millipore Corporation, MA, USA) to remove the salt. Finally, canola protein isolate (CPI) was freeze-dried (Labconco Corporation, Kansas City, Missouri 64132) at temperature difference of 35 ºC for 24 h to yield the CPI powder for later use.

The crude protein composition of CPI powder was determined using the Association of Official Analytical Chemists Method 920.87 (AOAC, 1995). The CPI produced was found to be comprised of 90.45% protein (%N x 6.25). CPI concentrations used in this study reflected the protein content rather than powder weight.

4.3.3 Preparation of canola protein isolate films

5.0% (w/w) CPI was dissolved in Milli-Q water under stirring at 500 rpm (IKAMAG RET-G, Janke & Kunkel GMBH & CO. KG, IKA-Labortechnik, Germany) to prepare film forming solution, which was then adjusted to pH 3.0 using 1 M HCl, and stirred for 1 h at room temperature. Because of the good water solubility, protein miscibility, and lower toxicity of glycerol, sorbitol, and polyethylene glycol 400 (PEG-400) (Barreto et al., 2003; Cao et al., 2009), they were then added at 50% (w/w of CPI) as plasticizers into the film forming solutions, and then allowed to stir (500 rpm) for an additional 10 min. The concentrations of CPI and plasticizers were decided based on the preliminary experiments. GP which is an effective naturally occurring cross linking agent was chosen because of its low cytotoxicity (Song & Zhang, 2009). A 0.4% (w/w) GP solution was created by dissolving GP (1% w/w of CPI) into Milli-Q water, and then added in the film forming solutions to stir (500 rpm) for 15 min. Gonzalez and co-workers (2011)

prepared soy protein films with different concentrations of GP (0.0%, 0.1%, 1.0%, 2.5%, 5.0%, 7.5%, and 10.0% w/w of soy protein), they found the films with 1.0% GP had better mechanical strength and water vapor barrier property compared with films with other concentrations of GP, because the cross linking reaction by small and large amounts of GP can take place intermolecularly and intramolecularly, respectively (Park et al., 2000). Since CPI and soy protein have similar amino acid profiles (Chabanon et al., 2007; Wang et al., 2008), 1.0% (w/w of CPI) GP was added into the film forming solution to investigate the effect of fixative condition on the properties of CPI films. Table 4.1 gives the contents of each film formulation tested. The film forming solutions were then degassed for 10 min within an ultrasonic bath at a frequency of 40 kHz (Branson Ultrasonic Cleaner, Model 2510R-DTH, USA) at room temperature. Afterwards, the film forming solutions were heated to 50 ºC under stirring at 500 rpm for 5 min, and then casted onto a polytetrafluoroethylene (PTFE) mould (10 cm length; 10 cm width; 0.10 mm depth). Excess film forming solutions were removed using a straight edge. CPI films were formed after drying overnight at room temperature. Films were then removed from the mould, and conditioned to 54% relative humidity (using a saturated magnesium nitrate solution) within a desiccator at room temperature for 2 d. All films were prepared in triplicate.

4.3.4 Film thickness

Film thickness was measured by using a digital micrometer (Model 62379-531, Control Company, U.S.A.) having a precision of 0.01 mm. Ten thickness measurements were taken on each triplicate film prepared.

4.3.5 Opacity

Film opacity was determined by using a spectrophotometer (Genesys 10uv, Thermo Fisher Scientific) as described by Gontard and co-workers (1994). The pre-conditioned films were cut into small strips (4.5 x 0.9 cm) and placed on the inside wall of the plastic cuvette (1 cm path length). The absorbance of film strips was measured at wavelength of 400 nm, 500 nm, 600 nm, 700 nm, and 800 nm. The area under the absorbance-wavelength curve was determined as the film opacity with the unit of A.nm. All measurements were performed in triplicate, for each type of films.

Table 4.1 Composition of CPI film forming solutions prior to film casting.

Film	CPI (g)	CPI (% db)	Plasticizer (g)	Plasticizer (%/CPI)	GP (g)	GP (%/CPI)	Water (g)	Thickness (mm)
5.0% CPI, 50% Gly	5	67	2.5	50	0.00	0	92.50	0.07 ± 0.01
5.0% CPI, 50% Sor	5	67	2.5	50	0.00	0	92.50	0.09 ± 0.01
5.0% CPI, 50% PEG-400	5	67	2.5	50	0.00	0	92.50	0.10 ± 0.01
5.0% CPI, 50% Gly, 1% GP	5	66	2.5	50	0.05	1	92.45	0.10 ± 0.01
5.0% CPI, 50% Sor, 1% GP	5	66	2.5	50	0.05	1	92.45	0.09 ± 0.01
5.0% CPI, 50% PEG-400, 1% GP	5	66	2.5	50	0.05	1	92.45	0.08 ± 0.01

4.3.6 Water vapor permeability

Water vapor permeability (WVP) of the CPI films was determined gravimetrically at room temperature (21-23^{o}C) using the "cup method" modified from ASTM standard method E96-93 (ASTM E96-93, 1993). For this study, PVC (polyvinyl chloride) cups were prepared to the following dimensions: outer cup height (2.65 cm), outer cup radius (2.50 cm), inner cup height (2.00 cm) and inner cup radius (2.25 cm). Film specimens were cut from each preconditioned film. Each specimen was sealed by a rubber O-ring to the PVC cup containing 10 mL of saturated $Mg(NO_3)_2$ solution (54% relative humidity). The entire cup (with $Mg(NO_3)_2$ solution plus film) was then placed within a desiccator containing $CaSO_4$ desiccant (0% relative humidity) at room temperature. The water vapor transmission rate though the film was determined from the weight loss of the system (cup plus $Mg(NO_3)_2$ solution) over a 5 h duration. The system (cup plus $Mg(NO_3)_2$ solution) was weighed to the nearest 0.1mg using an analytical balance (CPA224S, Sartorius, U.S.A.). Preliminary tests (not shown) showed that a steady state of weight loss was reached after 5 h. WVP values were calculated using the WVP Correction Method described by Gennadios and others (1994) as the following formulae.

$$WVP = \frac{WVTR_m \times L}{P_{w1} - P_{w2}} \qquad [3.1]$$

$$P_{w1} = P_T - (P_T - P_{w0})\exp\left(N_w h / cD\right) \qquad [3.2]$$

$$N_w = \left(6.43 \times 10^{-11}\right) \times WVTR_m \qquad [3.3]$$

where $WVTR_m$ (water vapor transmission rate, g/m^{2}s) was calculated by dividing the slope by the open area of the cup (15.90 cm^2); and L was the thickness of the film (mm). P_{w1} was water vapor partial pressure at the film inner surface (kPa), P_{w2} was the water vapor partial pressure at film outer surface (kPa), since the cup was placed in the desiccator containing $CaSO_4$ desiccant (0% relative humidity), and P_{w2} was 0 kPa. P_T was the total atmospheric pressure (101.3 kPa); P_{w0} was the partial pressure of water vapor in the air at the surface of the $Mg(NO_3)_2$ solution which was 1.34267 kPa; N_w (g.mol/s.cm^2) was the measured value of $WVTR_m$; h was the stagnant air gap height between the film and the surface of $Mg(NO_3)_2$ solution; c was the total molar concentration of air and water vapor (4.15×10^{-5} g.mol/cm^3); D was the diffusivity of water vapor through air at

25 ºC (0.25375 cm^2/s). All measurements were performed in triplicate for each type of films.

4.3.7 Mechanical properties

Tensile strength, tensile elongation and elastic modulus

Tensile strength (TS, MPa), tensile elongation (TE, %), and elastic modulus (E, kPa) of the film were determined using a Texture Analyzer with a load cell of 25 kg (Texture Technologies Corp., New York) on film strips (8 × 2.5 cm) which were pre-conditioned at 54% relative humidity under room temperature based on the ASTM D882-91 (1991). The film strips were placed between grips, and set up the initial grip separation to 40 mm and cross-head speed to 5 mm/s. The stress-strain curve data were collected by a microcomputer. TS was calculated by dividing the maximum load of the film strip by the area of cross-section of that strip (width of the strip (2.5 cm) × thickness of the strip); TE was calculated as a percentage of the length change of the film strip at the breakpoint of the film; E was expressed as the slope of the trend line on the stress-strain curve. Three measurements were taken on each triplicate film prepared.

Puncture strength and deformation

Both puncture strength (PS, N) and deformation (PD, mm) of the film were determined using a Texture Analyzer (Texture Technologies Corp., New York) as described by Gontard and other researchers (1992). Each film was stabilized on the puncture mould (65.6 mm diameter), and the smooth edged cylindrical probe (4 mm diameter) was placed just above the center of film and moved through the film at a cross-head speed of 1 mm/s. The force-deformation curve data were collected by a microcomputer. PS was calculated as the maximum force (N) which was loaded on the film to puncture the specimen. PD was expressed as the length changes at the rupture point of film.

4.3.8 Scanning electron microscopy (SEM)

Cross-sectional images of all CPI films were taken using a scanning electron microscope (Philips 505, Holland) operated at 30 kV. Specimens (0.5 cm × 0.5 cm) were cut and coated using a gold sputter coater (Edwards Sputter Coater S150B) in order to make samples conductive, and observed at 655 × magnification.

4.3.9 Statistical analyses

All experiments were performed on triplicate films and reported as the mean ± one standard deviation. A two-way analysis of variance (ANOVA) was used to measure statistical differences in thickness, opacity, WVP and mechanical properties (TS, TE, E, PS and PD) of CPI films among the various treatments (e.g., effect of plasticizer-type (glycerol, sorbitol and PEG-400) and fixative conditions (with and without GP)).

4.4 Results and discussion

4.4.1 Mechanical properties

Film strength

The effects of plasticizer-type and GP on the strength (PS, TS and E) of CPI films were examined and shown in Figure 4.1A, 4.2A and 4.2C, respectively. An analysis of variance of PS data indicated that plasticizer-type ($p<0.001$) and fixative conditions ($p<0.001$), along with their interaction ($p<0.01$) were all significant. Overall, the PS of CPI films prepared with GP was higher than those without, however the magnitude and changes in magnitude of PS differed slightly depending on which plasticizer was presented. Increase ratios of PS values by the addition of GP were 1.9x, 1.8x, and 1.9x for CPI films with glycerol, sorbitol, and PEG-400, respectively (Figure 4.1A). Films with sorbitol or PEG-400 displayed similar PS values ($p>0.05$), which were significantly higher than films prepared with glycerol (Figure 4.1A).

An analysis of variance on TS data indicated that both plasticizer type ($p<0.001$) and fixative condition ($p<0.001$) were highly significant, along with their interaction ($p<0.05$). Overall, TS of CPI films were greater in the presence of GP than without, however the magnitude and magnitude changes were dependent upon the plasticizer-type. For instance, the addition of GP resulted in 1.9x, 1.3x, and 1.8x increase of TS on films with glycerol, sorbitol, or PEG-400, respectively (Figure 4.2A), and films with sorbitol were stronger than films with PEG-400, followed by the films with glycerol (Figure 4.2A). In contrast to the other formulations examined, the addition of GP only led to an increase in TS of 1.3x suggesting sorbitol by itself was playing a more substantial role in enhancing film strength than the other plasticizer-types.

An analysis of variance of E data indicated that plasticizer-type ($p<0.001$) and fixative conditions ($p<0.001$), along with their interaction ($p<0.01$) were all significant. E data followed a

similar trend as TS, where overall, E of CPI films was greater in the presence of GP than without, however the magnitude and magnitude changes were dependent upon the plasticizer-type. Increase ratios of E values by the addition of GP were 2.5x, 1.3x, and 1.9x for CPI films prepared with glycerol, sorbitol, and PEG-400, respectively (Figure 4.2C). Films with glycerol were much weaker than films with sorbitol or PEG-400 (Figure 4.2C). Film thickness for all films ranged between 0.07 to 0.10 mm (Table 4.1), however they were not statistically different.

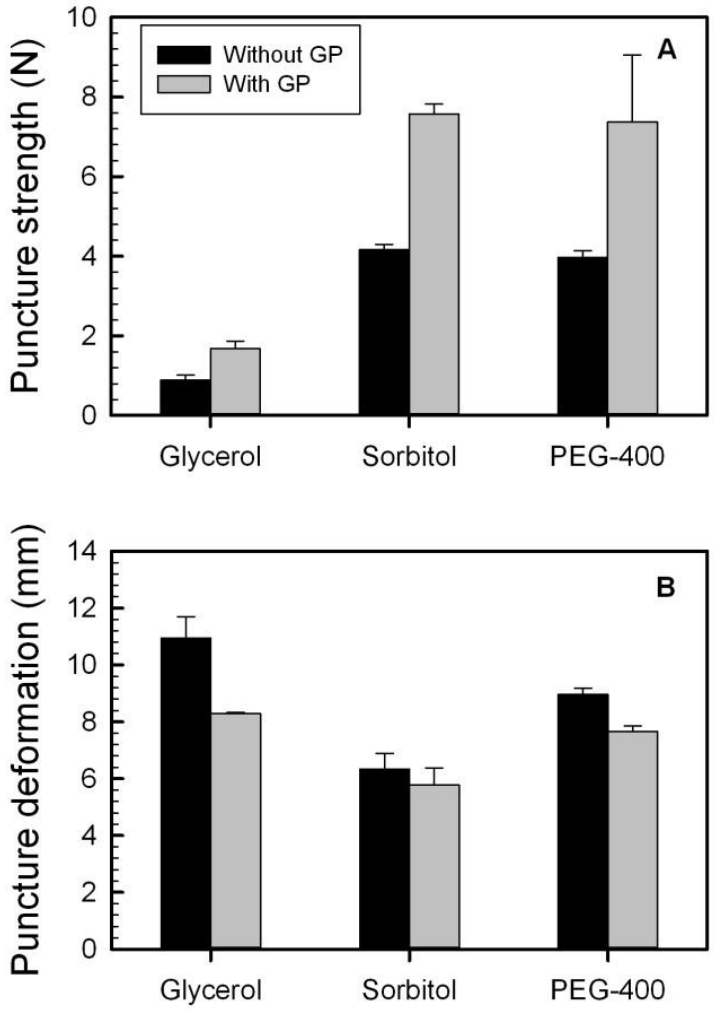


Figure 4.1 Puncture strength (A) and deformation (B) for 5.0% (w/w) canola protein isolate (CPI) films in the presence of 50% (w/w of CPI) glycerol, sorbitol, and PEG-400 prepared with and without 1% (w/w of CPI) genipin (GP).

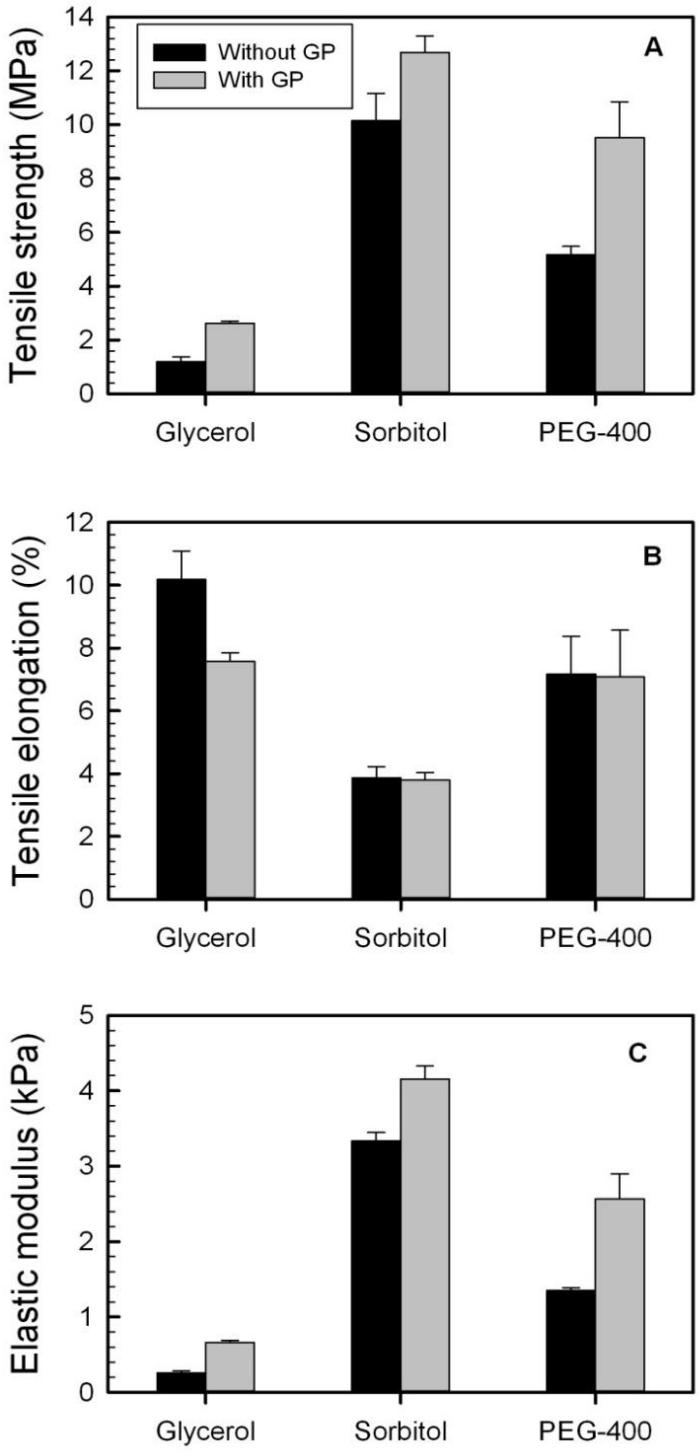


Figure 4.2 Tensile strength (A) and elongation (B), and elastic modulus (C) for 5.0% (w/w) canola protein isolate (CPI) films in the presence of 50% (w/w of CPI) glycerol, sorbitol, and PEG-400 prepared with and without 1% (w/w of CPI) genipin (GP).

In general, plasticizers are added to film forming solutions to overcome brittleness and increase flexibility associated with the protein-based film by modifying its structure. Some of stabilized protein-protein interactions within the film are replaced by plasticizer-protein interactions, leading to increases in void volume within the film and a rise in chain mobility in response to shear stress as the plasticizer disrupts the internal structure (Mangavel et al., 2003). Depending on the composition, size, and shape of the plasticizer added, varying abilities to modify structure can be observed (Sothornvit & Krochta, 2001). Theoretically, plasticizers containing more polar groups (-OH) should behave as better plasticizers due to the development of more protein-plasticizer interactions within the film, primarily via hydrogen bonding (Yang & Paulson, 2000a). However, molecular size, solubility, and polarity of plasticizers are other factors to impact the hydrogen bonding ability of plasticizers. Turhan and co-workers (2001) found that although polyethylene glycol with higher molecular weight has more polar groups, it has decreased hydrogen bonding ability, due to its decreased polarity and solubility. Therefore, the complexity of protein-plasticizer interactions and structure modification are important for mechanical strength of films. For instance, Ooi and co-workers (2012) reported polyvinyl alcohol/rambutan skin waste flour films prepared with glycerol led to lower TS than those prepared with sorbitol, since the glycerol was able to imbibe more water. Turhan et al. (2001) suggested that the plasticizers with higher molecular weight (e.g., PEG-4000 and PEG-8000) in methylcellulose-based films had reduced ability to form hydrogen bonds with the protein, leading to less plasticizing effect than lower molecular weight ones (e.g., PEG-400). Furthermore, the compatibility of the plasticizer to the protein, in terms of phase separation or physical exclusion between plasticizer and protein, can also impact its structure modifying abilities. For instance, Orliac et al. (2003) and Cao et al. (2009) compared the effect of PEG-400 with glycerol and sorbitol on sunflower protein-based films and gelatin films, respectively, and found PEG-400 molecules had lower compatibility to both of protein-based films.

In the present study, CPI films were overall stronger in the presence of sorbitol, than glycerol or PEG-400. It was hypothesized that sorbitol with six hydroxyl groups should has a better plasticizing effect than glycerol containing only three hydroxyl groups. In fact, the size of the glycerol was more compatible to the CPI network than the bigger sorbitol molecule, allowing it to disrupt protein-protein interactions better than sorbitol. Furthermore, glycerol with higher water affinity was able to attract more water molecules into the film via glycerol-water

interactions. In contrast, the PEG-400 polymer was proposed to be not as compatible as sorbitol with CPI, and would be less effective to insert itself in-between protein-protein interactions, due to its poor hydrogen bonding ability in the film structure. Therefore, addition of PEG-400 possibly resulted in phase separation in the film structure rather than homogenously dispersion.

Differences between strength (e.g., TS) among a selected few of protein-based films (e.g., soy and egg albumin) relative to those found in the present study, as a function of plasticizer-type are shown in Table 4.2. For instance, soy and egg albumin-based films prepared with sorbitol experienced a ~2.0 or ~2.8-fold increase, respectively relative to glycerol. However, CPI films prepared in the present study were ~8.5-fold stronger in the presence of sorbitol than glycerol (without GP) (Table 4.2). Differences among the various proteins may also depend on the level of denaturation induced during preparation of the film forming solution. Liu et al. (2004) found that the three-dimensional structure of protein-based film is more compact with higher levels of denaturation; leading to a stronger film. Unfolding to the protein's tertiary structure exposed buried hydrophobic amino acids that partake in hydrophobic interactions within the film matrix, and buried cysteine residues which undergo disulfhydryl exchange reactions to form stabilizing disulfide bridges. Consequently, the plasticizing effects can be reduced if the network structure is stronger (Kowalczyk & Baraniak, 2011). For instance, soy protein isolate films (Tang et al., 2005; Pruneda et al., 2008) reported in Table 4.3 were heated up to 70 ºC for 20 min, relative to the current study where CPI film forming solution was heated to 50^{o}C for 5 min. In contrast to work by Gennadios et al. (1996), in which TS data for egg albumin protein films prepared with sorbitol and PEG-400 were similar, the present study showed PEG-400 give CPI films reduced TS relative to those prepared with sorbitol. Similar results were reported by Orliac et al. (2003), Wan et al. (2005) and Cao et al. (2009) for sunflower protein films, soy protein films and gelatin films, respectively, where authors argued that PEG-400 displayed lower compatibility to the protein.

Table 4.2 Comparison of mechanical properties and water vapor permeability of protein-based films with different types of plasticizer.

Film type	Formulation	TS (MPa)	TE (%)	WVP ($g.mm/m^2.h.kPa$)
Soy protein[a]	5% SPI, 60% Gly	2.2 ± 0.3	159.9 ± 9.2	1.2 ± 0.0
	5% SPI, 60% Sor	4.2 ± 0.0	101.8 ± 15.6	1.2 ± 0.1
Soy protein[b]	5% SPI, 60% Gly	1.2 ± 0.2	186.9 ± 19.1	8.9 ± 0.1
	5% SPI, 60% Sor	2.4 ± 0.2	148.3 ± 9.7	5.3 ± 0.2
Egg albumin[c]	9% Egg albumin, 50% Gly	1.3 ± 0.1	32.2 ± 1.9	10.7 ± 0.3
	9% Egg albumin, 50% Sor	3.7 ± 0.2	15.0 ± 1.4	4.9 ± 0.2
	9% Egg albumin, 50% PEG-400	3.8 ± 0.2	59.7 ± 6.8	6.2 ± 0.2
Canola protein*	5% CPI, 50% Gly	1.2 ± 0.2	10.2 ± 0.9	1.2 ± 0.2
	5% CPI, 50% Sor	10.2 ± 1.0	3.9 ± 0.4	0.5 ± 0.1
	5% CPI, 50% PEG-400	5.2 ± 0.3	7.2 ± 1.2	0.9 ± 0.1

References: [a]Tang et al. (2005), [b]Pruneda et al. (2008), [c]Gennadios et al. (1996), *present study

Abbreviations: soy protein isolate (SPI); canola protein isolate (CPI), glycerol (Gly); sorbitol (Sor); polyethylene glycol 400 (PEG-400); tensile strength (TS) and elongation (TE); water vapor permeability (WVP)

Table 4.3 Comparison of mechanical properties and water vapor permeability of protein-based films with and without cross linking agents.

Film type	Formulation	TS (MPa)	TE (%)	WVP (g.mm/m^2.h.kPa)
(A) Genipin				
Soy protein[a]	8.33% SPI, 50% Gly	3.2 ± 0.1	22.5 ± 5.0	0.8 ± 0.0
	8.33% SPI, 50% Gly, 1% GP	4.2 ± 0.4	45.8 ± 0.3	0.6 ± 0.1
Canola protein*	5% CPI, 50% Gly	1.2 ± 0.2	10.2 ± 0.9	1.2 ± 0.2
	5% CPI, 50% Gly, 1% GP	2.6 ± 0.1	7.6 ± 0.3	1.4 ± 0.2
	5% CPI, 50% Sor	10.2 ± 1.0	3.9 ± 0.4	0.5 ± 0.1
	5% CPI, 50% Sor, 1% GP	12.7 ± 0.6	3.8 ± 0.2	0.5 ± 0.0
(B) Transglutaminase				
Soy protein[b]	5% SPI, 60% Gly	2.2 ± 0.3	159.9 ± 9.2	1.2 ± 0.0
	5% SPI, 60% Gly, 4 U MTGase	2.6 ± 0.3	105.9 ± 9.2	1.3 ± 0.1
	5% SPI, 60% Sor	4.2 ± 0.0	101.8 ± 15.6	1.2 ± 0.1
	5% SPI, 60% Sor, 4 U MTGase	4.5 ± 0.4	27.3 ± 3.6	1.3 ± 0.0
Wheat gluten[c]	5% WG, 40% Gly	1.1 ± 0.2	36.2 ± 5.2	-
	5% WG, 40% Gly, 8 U MTGase	1.4 ± 0.2	20.8 ± 2.5	-

References: [a]Gonzalez et al. (2011), [b]Tang et al. (2005), [c]Tang and Jiang (2007), *present study

Abbreviations: soy protein isolate (SPI); canola protein isolate (CPI); wheat gluten (WG); glycerol (Gly); sorbitol (Sor); genipin (GP); transglutaminase (MTGase); tensile strength (TS) and elongation (TE); water vapor permeability (WVP); unit (U)

In the present study, the addition of GP is presumed to form both inter- and intramolecular cross links to strengthen all CPI film structures, regardless of the plasticizer-type used. Although the exact mechanism of GP cross linking is unknown, it is believed to occur between ε-amine groups (e.g., mainly lysine, and to a lesser extent hydroxylysine and arginine) and different sites on the GP molecule via a nucleophilic attack reaction occurring as soon as GP and CPI film forming solutions mixed and a slower S_N2 nucleophilic substitution reaction. Butler et al. (2003) and Mi et al. (2003) proposed a mechanism involving GP attack on the amino containing cationic polysaccharide, chitosan. In brief, it involves a nucleophilic attack by a methylamine compound on the oleginic carbon at C-3 on deoxyloganin aglycone in the GP molecule causing the dihydropyran ring to open up. A second attack on the same amine group gives an aldehyde. The S_N2 nucleophilic substitution reaction between an amine group and the GP molecule leads to a replacement of the ester group on the GP molecule and release of a methanol molecule. Because of these two reactions, GP molecules can polymerize with each other to form chains up to 30-40 monomers in length, allowing them to partake in both short and long range cross linking (Liang et al., 2004).

Film deformation

The effects of plasticizer-type and fixative condition on the deformability (e.g., PD and TE) of CPI films were shown in Figures 4.1B and 4.2B, respectively. An analysis of variance of PD data indicated that both plasticizer-type ($p<0.001$) and fixative condition ($p<0.001$), along with their interaction ($p<0.01$) were highly significant. Overall, PD was found to be less with the addition of GP (~7.3 mm) than without (~8.8 mm); and PD was found to be the lowest for sorbitol (~6.1 mm) followed by PEG-400 (~8.3 mm) and then glycerol (~9.6 mm) (Figure 4.1B). However, the effect of GP on each film differed depending on the plasticizer present. For instance, CPI-sorbitol films only experienced a 1.1-fold decrease in PD data from ~6.3 to ~5.8 mm with the addition of GP, whereas CPI-PEG-400 and CPI-glycerol films experienced a 1.2-fold (decreasing from ~9.0 to ~7.7 mm) and 1.3-fold (decreasing from ~11.0 to ~8.3 mm) decline, respectively. An analysis of variance of TE data indicated that both plasticizer type ($p<0.001$) and fixative condition ($p<0.05$), along with their interaction ($p<0.05$) were significant. Plasticizer-type had a strong influence on the TE of the CPI films, more so than the presence of GP. TE values for CPI-sorbitol (~3.9%) and CPI-PEG-400 (~7.2%) films were similar regardless

of the presence of GP, whereas CPI-glycerol films significantly higher (~10.2%) in the absence of GP than with (~7.6%) (Figure 4.2B).

Overall, CPI films with different plasticizers prepared with and without genipin showed significantly reduced flexibility (e.g., % TE) relative to cross linked and/or uncross linked films prepared using soy protein (Tang et al., 2005; Pruneda et al., 2008), egg albumin (Gennadios et al., 1996) and wheat gluten (Tang & Jiang, 2007) (Tables 4.2 and 4.3). Theoretically, the formation of protein-based films involves inter- and intramolecular disulfide bonds and hydrophobic bonds (Okamoto, 1978). Under alkaline conditions, the reduction of disulfide bonds allows the complete protein dispersion, followed by the reformation of disulfide bonds through the air oxidation during drying, finally results in the reorganization of film structure to create the free volume within the film network. Since soy protein films, egg albumin films, wheat gluten films were prepared under alkaline conditions (pH 8-11), their structures supposed to be more flexible than CPI films which were prepared under acidic condition (pH 3). As previously described, plasticizers act to decrease intra- and intermolecular protein-protein interactions to increase void space in the film making it more flexible (Lieberma & Gilbert, 1973). Due to glycerol's hygroscopic nature, water molecules tend to be drawn into the film during its formation (Cheng et al., 2006). Films containing glycerol tend to be more flexible (higher %TE) than sorbitol, because glycerol can absorb more water molecules which is also a plasticizer (Gontard et al., 1993) in the film structure. The addition of PEG-400 was found to be incompatible to the protein-based films relative to glycerol or sorbitol, as previously described, resulting in an intermediate %TE value between films with glycerol and those with sorbitol.

The additions of fixatives function to counteract the effects of plasticizers by inducing intra- and intermolecular protein-protein cross links to make the films stronger and less flexible. Tang et al. (2005) reported that soy protein-glycerol and soy protein-sorbitol formulations formed stronger (e.g., increased TS) and less flexible films (e.g., lower %TE) with the addition of microbial transglutaminase, which is a natural enzymatic cross linking agent, relative to those without (Table 4.3). A similar trend was also reported by Tang & Jiang (2007) for wheat gluten-glycerol films with and without transglutaminase (Table 4.3). In the present study, CPI-glycerol films also followed this trend in the presence and absence of GP. However, although the addition of GP significantly increased film strength in CPI-sorbitol and CPI-PEG-400 films (Figure 4.2A), it did not significantly affect TE values (Figure 4.2B). The

similar result was also found on the chitosan film plasticized by polyethylene oxide (a molecular weight of 20,000 g/mol) with the addition of GP (Jin et al., 2004). The lower miscibility between plasticizer and biopolymer (e.g., CPI and chitosan) could be contributing to those results, therefore, the addition of GP is less effective to create the expansible networks in the films by breaking the protein-protein and/or protein-plasticizer interaction (Gonzalez et al., 2011). Gonzalez and co-workers (2011) found the presence of GP increased both TS and TE values in soy protein-glycerol films (Table 4.3). Differences in film behavior in the presence of GP may reflect differences in the level of GP polymerization and intra- and intermolecular cross linking occurring within the protein network, heterogeneously distributed around the plasticizer inclusions.

4.4.2 Film opacity

Film opacity is an important attribute in terms of food packaging, because transparency of packaging allows consumers to see the product before buying (Gontard et al., 1992; Orliac et al., 2003). In the present study, all of CPI films were natural yellow color, and plasticizers and GP didn't affect the color of films. Film opacity was investigated as a function of plasticizer-type and fixative condition and presented in Figures 4.3. An analysis of variance found only the main effects of plasticizer-type ($p<0.001$) and fixative condition ($p<0.001$) were significant, whereas their associative interaction was not ($p>0.05$). Overall, films prepared with glycerol were less opaque (~82.7 A.nm), followed by CPI-sorbitol (~94.3 A.nm) and CPI-PEG-400 (~102.6 A.nm) films (Figure 4.3). And the application of GP decreased transparency of films from ~100.1 A.nm to ~86.3 A.nm (Figure 4.3). Based on these findings, it was hypothesized that since the glycerol molecule was smaller than sorbitol and PEG-400, it was more homogenously dispersed. In contrast, both sorbitol and PEG-400 were more heterogeneously dispersed causing light to scatter more. A few studies (Orliac et al., 2003; Cao et al., 2009) also reported a "blooming" and "blushing" phenomenon could also occur on the surface of films plasticized by PEG-400, due to its lower compatibility with protein matrix, so, phase separation or physical exclusion could greatly increase the opacity of films. In contrast, cross linking with GP causes opacity to rise due to an increase in protein-protein interactions, and GP molecules competed with protein molecules to form covalent cross links to further disrupt the homogeneity of film. Therefore, the reduction of the degree of film network homogeneity led to the decrease of transparency of films. A rise of

opacity was also reported by Gonzalez et al. (2011) for soy protein films with GP, and by Tang et al. (2005) for soy protein films with transglutaminase. Ideally, the GP cross linking reaction induces a blue color once bound with proteins (Song & Zhang, 2009; Gonzalez et al., 2011), because of the spontaneous reaction of GP with amino acids in proteins (Touyama et al., 1994). However, in the present study, CPI films didn't turn to blue after the addition of GP. The small amount of GP and lower degree of denaturation of CPI could contribute to this result, so CPI didn't expose the enough reactive sites to form cross links with GP molecules. In addition, the high solubility of CPI films which was measured through the swelling index could further demonstrate this result. Theoretically, due to the formation of cross links by the addition of GP, the solubility of CPI films with GP supposed to be decreased. However, in the present study, CPI films with GP were dissolved into water immediately, because of the lower cross linking degree between GP and CPI. Since CPI films are highly soluble, they could be used for water soluble packets similar to cellulose ether-based packets.

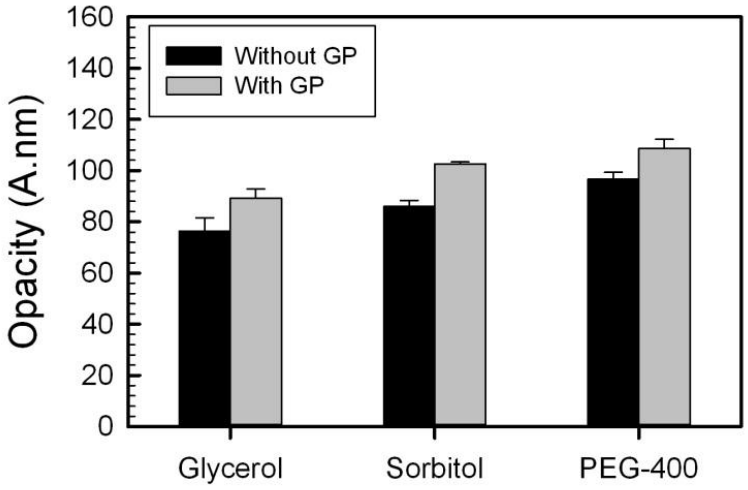


Figure 4.3 Opacity of 5.0% (w/w) canola protein isolate (CPI) films in the presence of 50% (w/w of CPI) glycerol, sorbitol, and PEG-400 prepared with and without 1% (w/w of CPI) genipin (GP).

4.4.3 Water vapor barrier property

The influences of plasticizer-type and fixative condition on WVP of CPI films were illustrated in Figure 4.4. An analysis of variance of WVP data found that only plasticizer-type was significant ($p<0.001$), whereas fixative condition ($p>0.05$) and their interaction term ($p>0.05$) were not. Overall, CPI-glycerol films showed the highest WVP (~1.3 g.mm/h.m^2.kPa), followed by CPI-PEG-400 (~0.9 g.mm/h.m^2.kPa) and CPI-sorbitol (~0.5 g.mm/h.m^2.kPa) films (Figure 4.4). The differences on WVP of films plasticized with glycerol, sorbitol, and PEG-400 could be caused by the different hygroscopic properties of the plasticizers. As reported in the study of water sorption equilibrium data by Rockland (1984), sorbitol exhibits lower absorptive properties than PEG-400, followed by glycerol. The hydrophilic nature of glycerol allows it to easily absorb more water molecules into films to increase the WVP. Furthermore, plasticizers of lower molecular weight can easily penetrate into the protein structure to disrupt the intermolecular interactions and increase the free volume of protein matrix; eventually increase the permeability of films (McHugh & Krochta, 1994; Sothornvit & Krochta, 2000). CPI-PEG-400 films were also presumed to have higher WVP than CPI-sorbitol films, due to the presence of a large number of hydroxyl groups (-OH) which increases its affinity to water (Wan et al., 2005). Similar findings as a function of plasticizer-type were reported in soy protein (Wan et al., 2005) and oat spelt arabinoxylan (Mikkonen et al., 2009) films. Tables 4.2 (p. 55) and 4.3 (p. 56) gave WVP data for various protein-based films. CPI-based films prepared within the present study showed comparable WVP data to those reported by Tang et al. (2005) for soy protein films with and without transglutaminase (~1.2 g.mm/h.m^2.kPa), and by Gonzalez et al. (2011) for soy protein films with and without GP (~0.7 g.mm/h.m^2.kPa) (Tables 4.2 and 4.3). In theory, higher degree of protein denaturation results in the great exposure of sulfhydryl groups and hydrophobic side chains which will reform during film drying process to promote cohesion of films, which lead to the better barrier properties of films. This is why soy protein films (prepared at 70 ºC for 2 h) by Gonzalez et al. (2011) had lower WVP than CPI films (prepared at 50 ºC for 10 min). However the CPI-based films were significantly better than films prepared with egg albumin (~4.9-10.7 g.mm/h.m^2.kPa) by Gennadios et al. (1996) and soy protein films (~5.3-8.9 g.mm/h.m^2.kPa) by Pruneda et al. (2008) (Table 4.2). Differences in amino acid profiles, proteins molecular properties, and film network could contribute to these results. This indicated that water vapor barrier property of films cannot be generalized, and it can be affected by the complex subjects

involving into the film preparation.

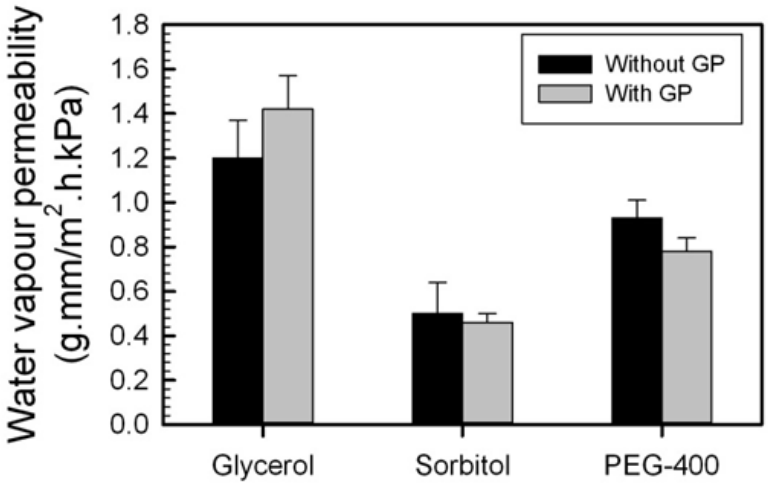


Figure 4.4 Water vapor permeability of 5.0% (w/w) canola protein isolate (CPI) films in the presence of 50% (w/w of CPI) glycerol, sorbitol, and PEG-400 prepared with and without 1% (w/w of CPI) genipin (GP).

4.4.4 Film morphology

Cross-sectional images of CPI films with and without GP, plasticized by glycerol, sorbitol, and PEG-400 were visualized by SEM (Figure 4.5). It was observed that some dispersed phase particles on each images (Figure 4.5). This implied that CPI film are agglomerates of CPI which linked together to form a continuous matrix. Overall, CPI films with GP (Figure 4.5, B1-3) had more compact, homogenous, and less porous structure than films prepared without GP (Figure 4.5, A1-3). The latter appeared more heterogeneous in nature with much larger pores. The smaller pore sizes in the presence of GP is hypothesized as the result of increased protein-protein interactions induced by intra- and intermolecular covalent cross linking; resulting in films that have increased mechanical strength. CPI-glycerol films (Figure 4.5, A1) showed a more organized structure with much larger pore size than CPI-sorbitol films (Figure 4.5, A2). Since WVP can be elevated by the greater size and larger amount of pores in the film structure to allow more water vapor to pass through the films, CPI-glycerol films had higher WVP than CPI-sorbitol films. The latter also showed a regular alignment of protein-protein aggregates with relatively smaller pores which may help explain its improved film strength and reduced

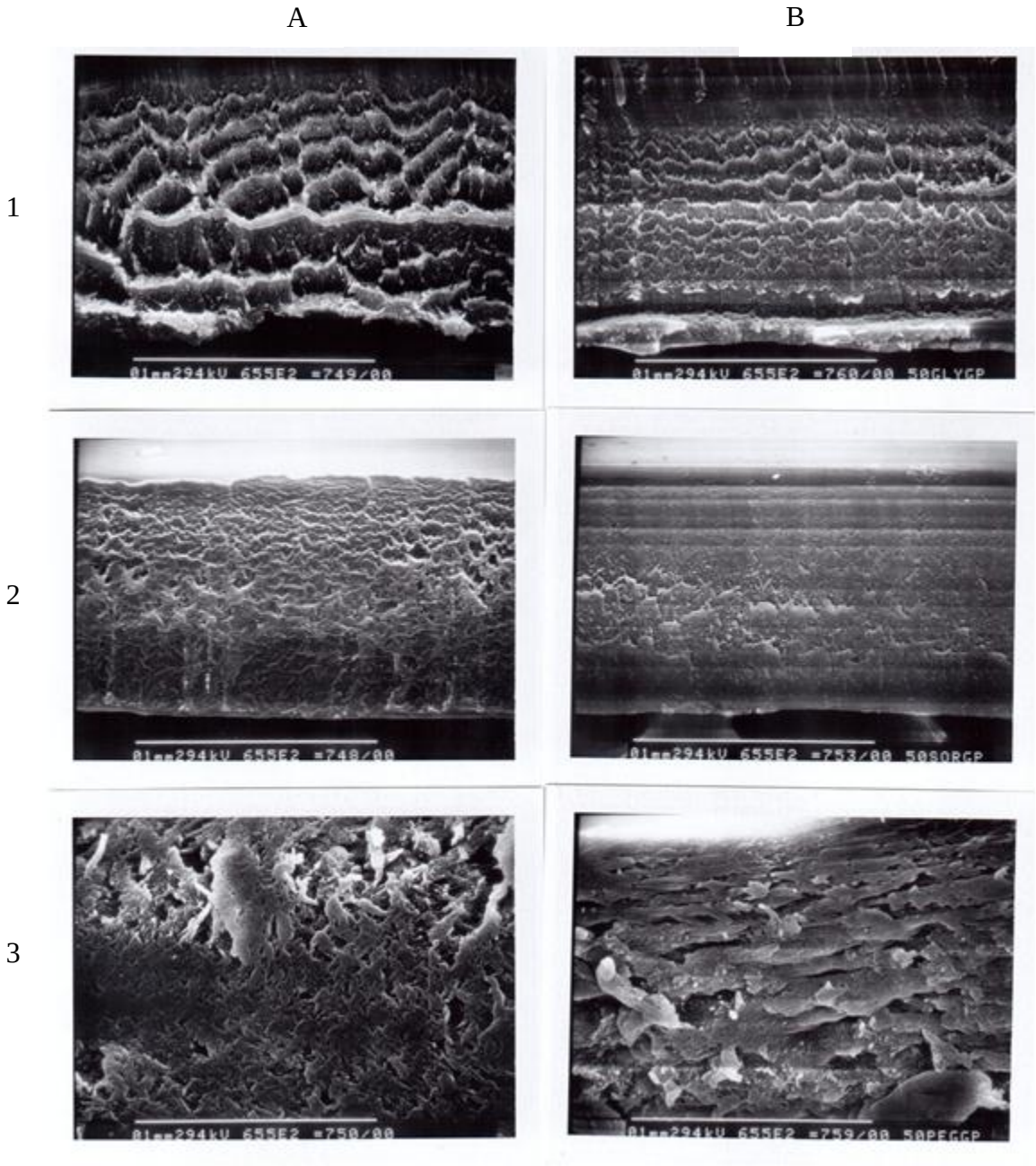


Figure 4.5 SEM cross-sectional images (at 655 × magnification) of 5.0% (w/w) canola protein isolate (CPI) films in the presence of 50% (w/w of CPI) glycerol (1), sorbitol (2), and PEG-400 (3) prepared in the absence (A) and presence of 1% (w/w of CPI) genipin (B).

flexibility. In contrast, CPI-PEG-400 films (Figure 4.5, A3) showed evidence of a more coagulated structure with large aggregates and different pore sizes. However the protein matrix looked less ordered than seen for CPI-sorbitol (Figure 4.5, A2) films and CPI-glycerol (Figure 4.5, A1) films; possibly reflecting the lower compatibility of PEG-400 with proteins in the film matrix. Because films with higher degree of homogeneity supposed to have improved mechanical properties, CPI-sorbitol films were stronger than CPI-PEG-400 films.

4.5 Conclusions

The present study evaluated the effect of plasticizer-type and fixative condition on the mechanical, optical and water vapor barrier properties, and morphology of CPI films. Generally, as the plasticizer changed from sorbitol to PEG-400, followed by glycerol, films became more flexible, and more permeable to water vapor. In contrast, when genipin was applied into films, films became stronger, less malleable, and more opaque. Based on these findings, CPI shows promise as a potential material for use in designing edible, biodegradable packaging in the future.

5. GENERAL DISCUSSION

Although canola proteins have been studied in terms of their functional properties (Aluko & McIntosh, 2001; Yoshie-Stark et al., 2008), efforts have not been made to explore the utilization of canola proteins into food products until recently. A few companies (e.g., BioExx Specialty Proteins (Toronto, ON, Canada) and Buron NutraSciences (Vancouver, BC, Canada)) started to bring canola protein into the marketplace as a new food ingredient. In order to help diversify the potential applications and markets for canola proteins, the film forming materials were investigated. The overall goal of this research was to design a canola protein isolate (CPI)-based film that provides excellent water vapor barrier, optical and mechanical properties. Specifically, the effect of protein concentration, glycerol concentration, plasticizer-type, and the addition of genipin on the mechanical, optical and water vapor barrier properties of CPI-based films were studied.

The formation of edible films using plant proteins have been previously reported using proteins from soy (Cho & Rhee, 2004), sunflower (Orliac et al., 2002), lentil (Bamdad et al., 2006), faba bean (Saremnezhad et al., 2011), pea (Choi & Han, 2001; Kowalczyk & Baraniak, 2011) and rapeseed (Jang et al., 2011). Plasticizers, such as glycerol, are typically added in the film forming solution to overcome brittleness issues associated with films, making them more malleable by replacing protein-protein interactions with protein-plasticizer interactions. As a result, the free volume within the film structure is increased, leading to a heterogeneous distribution of junction zones within the matrix (Guilbert, 1986; Kester & Fennema, 1986; Sothornvit & Krochta, 2005; Janjarasskul & Krochta, 2010). The structure modifying ability of plasticizers on the film structure is dependent on the composition, size, shape, and concentration of plasticizer used in the film forming solution (Sothornvit & Krochta, 2001). Moreover, the formation of cross links within the film through the addition of cross linking agents can further improve the strength and barrier properties, allowing the film to withstand the external stress and moist environment that could be happened during the production (Yang & Paulson, 2000b). For example, genipin, a natural chemical cross linking agent, could be added in the film forming

solution to react with primary amine groups of protein via a nucleophilic attack reaction and a slower S_N2 nucleophilic substitution reaction to form the inter- and intramolecular cross links in the film matrix to improve the properties of films (Muzzarelli, 2009; Gonzalez et al., 2011; Liu et al., 2012).

In the present study, 7.5% CPI films were found to be stronger than 5.0% CPI films presumed because of the increase of intermolecular interactions (e.g., hydrophobic interactions, and hydrogen bonding) within the film matrix and the greater thickness. Rhim et al. (1999) and Cho & Rhee (2004) reported a similar trend in protein concentration for soy protein-based films, which the authors attributed to increased biopolymer ordering within the film.

In the present study, CPI films were found to be more flexible but weaker with the increase of glycerol concentration. Glycerol acts to disrupt the order of protein-protein aggregates and replace protein-protein interactions by protein-glycerol interactions via hydrogen bonding, resulting in more heterogeneous spatial distribution of junction zones in the film structure (Gontard et al., 1993; Sothornvit & Krochta, 2005; Janjarasskul & Krochta, 2010). Choi & Han (2001) reported a similar trend as in the present study for TS and TE as a function of glycerol concentration, where TS decreased from ~4.9 MPa to ~0.7 MPa, but TE increased from ~0.6% to ~92.0% as the glycerol concentration increased from 20% to 50% in pea protein isolated-based films. However, Gennadios and co-workers (1993) found that soy protein-based films were more flexible when they were prepared under alkaline (pH 7.0 to 11.0) conditions than the films prepared under acidic (pH 1.0 to 3.0) conditions. Since, the present CPI films were prepared under acidic conditions (pH 3.0), they were less malleable than other plant proteins-based films (e.g., soy protein-based film and lentil protein-based film) prepared at alkaline conditions (pH 10.0 to 11.0) (Rhim et al., 1999; Bamdad et al., 2006).

Although plasticizers are typically applied to overcome the brittleness issues associated with films, the structure modifying ability is dependent on its composition, size, and shape of plasticizer added (Sothornvit & Krochta, 2001). In the present study, CPI films were overall stronger and less flexible in the presence of sorbitol, than glycerol or PEG-400, because sorbitol is smaller in size and more compatible to the film matrix than PEG-400, and is less hydrophilic than glycerol, attracting less water molecules into the film (Turhan et al., 2001; Ooi et al., 2012). This trend was also reported to occur for soy protein and egg albumin-based films (Gennadios et al., 1996; Tang et al., 2005; Pruneda et al., 2008). In the present study, CPI films were 9-times

stronger in the presence of sorbitol than glycerol (without genipin), but other films prepared with sorbitol only experienced ~2 or ~3-fold increase in film strength relative to glycerol. Liu and co-workers (2004) reported that the structure of protein-based films is more compact with higher levels of denaturation; leading to a stronger film. Consequently, the plasticizing effects can be reduced if the network structure is stronger (Kowalczyk & Baraniak, 2011). For instance, soy protein-based films (Tang et al., 2005; Pruneda et al., 2008) were heated up to 70ºC for 20 min, relative to the current study where the CPI film forming solution was heated to 50ºC for 5 min, therefore, sorbitol had less effects in soy protein-based films than in CPI films. Moreover, because of the formation of short and long range cross links in the film structure by the addition of genipin, CPI films with 1% genipin were stronger and less malleable than films without genipin. Theoretically, the cross linking reaction is believed to occur between ε-amine groups and genipin molecule via a nucleophilic attack on oleginic carbon and a slower S_N2 nucleophilic substitution reaction, in which the ester group on genipin is replaced to release a methanol molecule (Butler et al., 2003; Mi et al., 2003). A similar trend was also reported by Tang et al. (2005) for soy protein-based films and Tang & Jiang (2007) for wheat gluten-glycerol films with and without microbial transglutaminase.

Film opacity is an important factor to consider when designing food packages. It was found that CPI films were more opaque with higher levels of CPI present, since the total amount of solids present in the film was higher, greater ordering was occurring and film thickness was greater. In contrast, CPI films became more transparent with the increase of glycerol concentration, however the distribution of glycerol within the film was still presumed to be impacted by the high protein concentration presented. Since glycerol is much smaller than sorbitol and PEG-400, it was more homogeneously dispersed causing light to scatter less, and CPI films with glycerol were more transparent than films with sorbitol and PEG-400. In addition, a few studies (Orliac et al., 2003; Cao et al., 2009) found a “blooming” and “blushing” phenomenon could occur on the surface of films plasticized by PEG-400, due to its lower compatibility with protein matrix. In the current study, CPI films with PEG-400 were more opaque than the films with sorbitol. Furthermore, the formation of cross links through the addition of genipin resulted in a more compacted film structure which was demonstrated by SEM images of CPI films (Figure 4.5). Consequently, CPI films with 1% (w/w of CPI) genipin showed higher opacity than the films without. A similar finding was also reported by Gonzalez et

al. (2001) for soy protein films with genipin, and by Tang et al. (2005) for soy protein film with transglutaminase.

Water vapor permeability (WVP) of prepared films is another important factor to consider when designing food packaging, because it greatly affects the quality of food products. In the current study, WVP was found to increase with the increase of both protein and glycerol concentration, since protein-glycerol interactions were able to replace protein-protein interactions to increase the free volume in the film structure, causing greater influx of water (Gontard et al., 1993; Yang & Paulson, 2000a). In addition, the water adsorption ability of films can be raised by adding higher levels of hydrophilic materials (e.g., glycerol) in the film formulation (Kamper & Fenema, 1984), or by raising the protein content to allow a greater amount of protein-water interactions to occur (Jang et al., 2011). The trend in the present study is similar to the findings of Jang et al. (2011) for rapeseed protein films. However, WVP of CPI films was much lower in comparison with other plant protein films. This may be the result of a number of potential factors, such as film thickness, protein hydrophobicity, and relative humidity (RH) for WVP measurement. A few studies (Rhim et al., 1998; Choi & Han, 2001; Bamdad et al., 2006) reported that WVP for soy protein, lentil protein, and pea protein-based films was much higher under ~75% RH than the present CPI films under ~54% RH. Kokoszka and co-workers (2010) demonstrated for soy protein-based films that WVP for films at ~70% RH was much higher than films at ~23% RH. In the present study, it was also found that WVP of CPI films with glycerol was much higher than films with PEG-400, followed by films with sorbitol. Theoretically, water absorptive ability of sorbitol was much lower than PEG-400, followed by glycerol (Rockland, 1984). Glycerol has much lower molecular weight, allowing it to easily penetrate into the film matrix to disrupt the intermolecular interactions and increase the free volume of film structure (McHugh & Krochta, 1994; Sothornvit & Krochta, 2000). CPI films with sorbitol showed lower WVP than films with PEG-400 or glycerol. Similar findings as a function of plasticizer-type were reported in soy protein (Wan et al., 2005) and oat spelt arabinoxylan (Mikkonen et al., 2009) films. Moreover, WVP of CPI films should be decreased by the addition of genipin, because film structure was more compact by the formation of cross links, with less free volume present in the film matrix. CPI films prepared within the present study showed comparable WVP data to those reported by Tang et al. (2005) for soy protein films

with and without transglutaminase, and by Gonzalez et al. (2011) for soy protein films with and without genipin.

Since film structure is an important factor to decide the properties of films, film morphology was studied in this research by taking SEM images on the cross-section of CPI films plasticized by glycerol, sorbitol, and PEG-400 with and without genipin. Because of the formation of intra- and intermolecular cross links by the addition of genipin, CPI films with genipin had more compact and less porous structure than films without genipin. This is why CPI films with genipin had higher mechanical resistance and lower flexibility than films without genipin. CPI-sorbitol films showed a regular alignment of protein-protein aggregates with relatively smaller pores than CPI-glycerol and CPI-PEG-400 films to explain why CPI-sorbitol films had better moisture barrier property and mechanical strength. In contrast, CPI-PEG-400 films showed a more coagulated structure with large aggregates to reflect the lower compatibility of PEG-400 with proteins in the film matrix.

Film forming conditions for the CPI in the present study was restricted to acidic conditions (pH 3.0) and heating to 50^{o}C. Preliminary experiments found difficulty completely dissolving CPI under alkaline conditions (pH 8.0), most likely since it was close to the pI of cruciferin proteins within this isolate (pH 7.25). However, at pH 3.0, CPI at the concentration used was found to be completely soluble. Comparing with other plant protein films (e.g., those from soy, pea and lentil), CPI films showed better water vapor barrier properties and comparable strength, however were less flexible. These differences seen with other films relative to the CPI films may reflect the pH of the film forming solution. For instance, under alkaline conditions, the proteins would be highly charged allowing for greater affinity to water (hence poorer barrier properties) (Frinault et al., 1997), and display greater electrostatic repulsion. The latter would likely restrict aggregate growth to produce a film with a finer protein strand microstructure, and hence more mallable (Zirbel & Kinsella, 1988).

Moreover, temperature of the film forming solution can impact film properties. For instance, during preliminary experiments, film forming solutions heated to temperatures above 50^{o}C resulted in the formation of a gel. Therefore film forming solutions were only heated to 50^{o}C during this research to allow partial protein denaturation to occur, but still enable the solution to be easily poured and spread into the casting mould. At high temperatures, considerable protein denaturation typically occurs leading to increased levels of protein

aggregation via hydrophobic interactions, and depending on the material, covalent disulfide bonding could also occur (Anker et al., 1999). In general, films with a greater amount of protein denaturation typically lead to stronger films (Anker et al., 1999). According to Folawiyo & Apenten (1996), a cruciferin-dominated isolate starts to denature at 50 ºC under acidic condition (pH 3.0). Therefore, CPI films in the present study may have comparable or reduced mechanical properties than other plant proteins-based films which were formed under much higher temperature with longer time (Table 3.2).

Studies in literature also tend to differ with respect to the RH used the conditioning step and to run the WVP tests. RH differences result in the moisture adsorption of dry materials involving the binding of water molecules to specific hydrophilic sites (e.g., amino and hydroxy residues) of protein-based films, and swelling or conformational changes may also accompany with the adsorption in the film structure (D'Arcy & Watt, 1981; Watt, 1983).

6. GENERAL CONCLUSIONS

Overall, the present study investigated the influence of protein and glycerol concentration, plasticizer-type, and fixative condition on the mechanical, optical, and water vapor barrier properties of CPI films. In general, CPI films had higher mechanical strength as protein concentration increased due to the increased film thickness and a greater amount of intermolecular interactions occurring within the film structure. In contrast, as the glycerol concentration increased, CPI films became more flexible but weaker, presumably caused by protein-protein interactions being replaced by protein-glycerol interactions, and a more heterogeneous spatial distribution of junction zones within the film. Moreover, plasticizer-type is also an important factor to impact the mechanical properties of CPI films. CPI films were more flexible in the presence of glycerol, followed by sorbitol or PEG-400, since its smaller size was more compatible to the film matrix and its higher hydrophilic nature allowed it to attract water molecules which also performed as another plasticizer in the film structure; however, glycerol resulted in higher WVP of CPI films in comparison with sorbitol and PEG-400. Because of the formation of short and long range cross links in the film structure by the addition of genipin through the nucleophilic attack and S_N2 nucleophilic substitution reaction, CPI films became stronger but less malleable.

Film opacity was also studied as a function of protein and glycerol concentration, plasticizer-type and fixative condition in the film matrix. CPI films were more transparent as the glycerol concentration increased, because of the transparent nature and homogenous dispersion of glycerol in the film structure. However, films became more opaque at the higher CPI level presumed due to the higher solid contents, a more tightly packed structure, and greater thickness. Since glycerol (molecular weight of 92.09 g/mol) (Redl et al., 1999; Cunningham et al., 2000) is much smaller than sorbitol (molecular weight of 182.17 g/mol) (Barreto et al., 2003) and PEG-400 (molecular weight of 400 g/mol) molecules, it was presumed to be more homogeneously dispersed within the film forming solution, so, CPI films prepared with glycerol had lower opacity than films with sorbitol or PEG-400. In addition, due to the lower

compatibility of PEG-400 with the protein matrix, CPI films with PEG-400 were more opaque than films with sorbitol. Furthermore, the addition of genipin led to more opaque films than those without by the formation of cross links.

The effects of protein and glycerol concentration, plasticizer-type and the addition of genipin on WVP were also investigated in this research. WVP increased with the increase of both CPI and glycerol concentrations. In the case of glycerol, protein-protein interactions were replaced by the protein-glycerol interactions, leading to increases in free volume within the film to allow for a greater influx of water. In addition, higher levels of hydrophilic materials (e.g., glycerol and CPI) in the film formulation resulted in the increase of water mobility through the film matrix. Moreover, CPI films with sorbitol had lower WVP than films with PEG-400 or glycerol, because of the different water absorptive ability and molecular weight of plasticizers. CPI films with genipin were found to have lower WVP than without when sorbitol and PEG-400 were presented, however the same was not true when glycerol was present.

In this case of plasticizer-type and the addition of genipin in CPI films, film morphology was investigated by taking SEM images to explain the differences on the properties of CPI films. SEM images showed that CPI films with genipin had more compact and less porous structure than films without genipin to explain their better mechanical strength and water vapor barrier property. CPI-sorbitol films showed a more alignment structure with smaller pores than CPI-glycerol and CPI-PEG-400 films to explain their better mechanical resistance and lower moisture permeability. However, CPI-PEG-400 films had a more coagulated structure with larger aggregates to reflect the poor compatibility of PEG-400 with proteins.

In summary, although CPI film forming conditions (e.g., pH and temperature) were limited and the flexibility of CPI films was lower, CPI films had much better water vapor barrier properties and comparable film strength relative to other plant protein-based films, therefore, CPI shows promise as a potential material for the development of edible films/packaging in the future.

7. FUTURE STUDIES

With constantly growing public concerns over the large quantity of food packaging waste in our landfills, market trends continue to shift away from synthetic towards biodegradable edible materials. Biodegradable edible films have the potential to be used as wraps or added into bags or pouches to improve recyclability of the packaging system (Hernandez-Izquierdo & Krochta, 2008). Specifically, they can be used inside of foods as a barrier to migration between different layers, such as in pies and confectionery; or can be used as a controlled-release carrier to deliver antioxidants and antimicrobials, as well as minerals and vitamins to increase nutritional value of foods (Vargas et al., 2008). Protein-based films provide excellent properties to fulfill consumers' demands and expectations as a substitute to conventional synthetic petroleum-based food packaging. For instance, wheat gluten and soy protein films have been explored as a replacement for collagen in sausage casings. Due to the high solubility, soy protein films have also been used in the production of water soluble pouches (Krochta, 1997). Since canola proteins represent a new potential material for film production, a greater understanding on their film properties are needed, as well as optimization of their film forming conditions.

Despite polysaccharides- (e.g., starch, chitosan, and pectin), proteins- (e.g., soy proteins, whey proteins, and gluten) and lipids- (e.g., beeswax, resin, and candelilla wax) based films have been developed, and their properties have also been discussed over the past few decades, composite films which have combined advantages from polysaccharides-, proteins-, and lipids-based films have not gained many attentions in the food packaging industry. Therefore, research efforts in the future should be focused on the design of composite films by using CPI with both lipid and polysaccharide to take specific beneficial characteristics from each group to further diminish the drawbacks of single material-based films (Greener & Fennema, 1989). In general, one type of composite film could be developed by creating bi-layer type films, in which CPI layer is casted first, followed by a lipid layer, or creating CPI film using an emulsion, derived using a stable lipid-CPI emulsion (Krochta, 1997; Shellhammer & Krochta, 1997; Perez-Gago & Krochta, 2005). As such, CPI could combine with a hydrophobic material, such as

beeswax and resin, to improve the water vapor barrier properties of CPI-based films further.

Furthermore, CPI films could be produced without the need for cross linking agents (e.g., genipin) using a composite material involving both CPI and polysaccharides, at a pH where complex coacervation can occur. Complex coacervation occurs when two biopolymers of opposing net charges interact via electrostatic attractive forces to form electrostatic cross links. This typically occurs over a narrow pH range, at pH<pI of the protein (giving a net positive charge) and at pH>pK_a of the reactive group on the polysaccharide backbone (giving a net negative charge) (Janjarasskul & Krochta, 2010). Klassen and co-workers (2011) investigated CPI-alginate interactions as a function of pH (1.5-7.0) and biopolymer weight mixing ratio (1:1-50:1, w/w) by turbidimetric analysis to find a 20:1 CPI-alginate ratio at pH 4.5 was optimal for the complex coacervation. Such findings could be applied to alter the film forming solutions to create CPI-alginate composite films with unique functionality to CPI alone.

In the future, further optimization of the intrinsic and extrinsic factors involved with the process of producing CPI-based films is needed. The intrinsic factors are determined by the nature of proteins which includes macromolecular structure and configuration to influence protein-protein interactions in the film matrix during film formation process (Miller & Krochta, 1997). However, those interactions can be manipulated by controlling the film forming conditions (e.g., pH, temperature, and relative humidity (RH)) and the addition of additives (e.g., plasticizers and cross linking agents) which are considered as extrinsic factors (Sanchez et al., 1998). CPI film forming conditions were very limited on pH and temperature in the present study; as well CPI was only prepared using one extraction protocol (Folawiyo & Apenten, 1996; Klassen et al., 2011). Since, the extraction protocols can have a big impact on protein functionality; other prepared CPI materials could be tested to see the effect of protein processing on their film forming abilities. The pH of film forming solution is another very important factor to consider, because pH not only affects the solubility of proteins but can influence the level of protein-protein aggregation and electrostatic repulsion within the film, impacting water absorption, mechanical properties and microstructure of films (Anker et al., 1999). For example, Anker and co-workers (1999) found that a whey protein isolate film formed at pH 9.0 was more extensible than if formed at pH 7.0. However, since the CPI films are intended for use in edible packaging to improve quality and prolong shelf-life of food products, alkaline conditions for film formation is undesirable. The absence of pH adjustments to the film forming solution could be a

new trend in the film formation process (Kowalczyk & Baraniak, 2011). Furthermore, temperature and time of the heating process is critical to optimize, as it controls the level of protein denaturation within the system (Anker et al., 1999). Therefore, mechanical properties and permeability of films are partially determined by the temperatures for the protein denaturation, drying process, and film storage. For instance, Choi & Han (2002) found that pea protein isolate films prepared at 90 ºC for 20 min were much stronger than the films prepared without a heating step. Menegalli et al. (1999) observed that increasing air temperature during the drying process decreased drying kinetics for gelatin films, because of the structure changes around the sol-gel transition of the system (Sobral et al., 2001). Therefore, CPI films could be prepared on a higher temperature to improve the properties of films. Since CPI-based films are able to absorb water molecules, the RH of the conditioning step could have a big impact on film performance; especially water is an excellent plasticizer (McHugh & Krochta, 1994). Theoretically, the increased flexibility of films which are conditioned under high RH can be related to the increase of moisture content in the film matrix, where increased protein mobility could increase film flexibility and decrease strength (Donhowe & Fennema, 1992). Anker and co-workers (1999) found tensile elongation increased and strength decreased on whey protein isolate (WPI)-based films with increased RH during film conditioning. Overall, the effect of extrinsic factors (e.g., pH, temperature, and RH) on properties of CPI films which could be prepared by CPI from different extraction methods could be investigated in the future to better understanding and preparing CPI-based films.

Furthermore, some properties (e.g., gas permeability, color) of CPI films were not studied in the present research. Food ripening and rancidity are greatly affected by oxygen; therefore, the ability to control gas exchanges, particularly oxygen and carbon dioxide, is an important property of CPI films in terms of food packaging (Debeaufort et al., 1998). Oxtran is the most commonly used apparatus to test gas permeability of edible films (ASTM D3935, 1981). However, since this technique cannot measure gas permeability of films under different relative humidity which could happen during the storage of food products, gas chromatographic method has been developed (Liebermann et al., 1972; Hagenmaier & Shaw, 1992). Therefore, gas permeability of CPI films could be measured using gas chromatographic method. In addition, the changes to a food products' color with the addition CPI films should be considered, because it will influence consumer perception of quality. Generally, the color of films are measured by

using colorimeters and expressed as luminosity, chroma, and hue (Hutchings, 1999). For example, the changes on luminosity are related to the reflection changes on surface of sample after films are applied (Vargas et al., 2008). Although CPI films had light yellow color, different additives (e.g., plasticizers and cross linking agents) could still impact the color of CPI films, the study on the color of CPI is still necessary.

In the current study, the protein-based films were prepared using CPI extracted from canola meal, and therefore assumed to be biodegradable. Since CPI films were considered as an alternative to synthetic petroleum-based packaging, biodegradability of CPI films is very important to concern for the environmental protection. However, due to the addition of other materials, such as genipin, to create cross linking structure in the CPI film network, the biodegradability or the efficiency of biodegradability of CPI films may be influenced. For example, Gonzalez et al. (2011) found that the degradation of soy protein-based films in soil were greatly affected by the degree of cross linking, where higher degree of cross linking generally resulted in a longer degradation time of the films. Therefore, the biodegradability of CPI films could be measured using indoor soil degradation method in which CPI films would be dried and buried in a characterized soil to calculate film weight loss (%) after 35 d (Gonzalez et al., 2011).

In conclusion, CPI films showed potential as a suitable alternative to other plant protein-based films in the present study, however, more research surrounding the film forming conditions (e.g., pH, temperature, and RH), the formation of CPI-based composite films, and other properties of CPI films (e.g., gas permeability, color, and biodegradability) may be also necessary to better understand and prepare CPI-based films.

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